

Britain's discord on Europe's money

Britain is rehearsing again the role of black sheep at Europe's next intergovernmental conference, running the risk of alienating potential allies and perpetuating confusion among its voters.

A YEAR in advance of events for a change, Britain seems to be bent on opting out of any decisions taken by the Intergovernmental Conference of the European Union (EU) planned for next year. The explanation is simple; the governing Conservative party cannot agree within itself on European policy and especially on Britain's adoption of the proposed common currency for Western Europe. The prime minister, Mr John Major, although an enthusiast for European collaboration in the past, is now delicately poised between the sceptics who will have no truck with a common currency and those who believe its coming is inevitable; he has taken to saying that the project is unattainable in 1997 (the first date specified in the Maastricht Treaty), but not where he stands on membership at a later stage.

Thus threatens a tragicomedy, at least for Britain. The original plan for next year's conference was that it should make a start on strengthening collaboration between European countries in political and military affairs. No good purpose will be served by letting it be hi-jacked by arguments over a common currency, especially if they consist of restatements of British ambivalence. It is not as if the other issues can be left on ice. Just in the past week, EU member states (Britain included) have been agonizing over immigration policy, for example, while the noises from the United States that Britain should further reduce the striking capacity of its Trident nuclear submarines creates an urgent need for an Anglo-French understanding on strategic policy within the Western European Union.

On the common currency, British confusion mirrors abiding ignorance everywhere. The Maastricht Treaty is explicit about the conditions that potential users must meet before they can belong. There are upper limits on the rate of inflation, on the public sector deficit as a percentage of gross national product and on government debt (by the same yardstick). Otherwise, it has been agreed that there should be a central bank (called the European Monetary Institute), which will be politically independent of member governments. Its chief function will be to manage the foreign (or non-European reserves) that back the currency and to determine monetary policy (including interest rates) for the whole of Europe. But there will yet be endless argument about the institute's terms of reference as well as about the continuing obligations of member governments.

British politicians are right to say it is inconceivable that these questions, which are political as well as technical, can be settled in time for a start on a common currency by 1997.

One difficulty is that there is no obvious mechanism by which countries that run into economic difficulties can be rescued by the centre. But if there were, there would be no obvious way of preventing wayward governments from mismanaging their affairs in such a way as to invite rescue. That is the awkward dilemma that leads Britain's Eurosceptics to fear that a common currency would lead inexorably to a federal Europe. And it is difficult to see how these issues could be managed over the long haul without a greater degree of political cohesion than at present.

The other side of the coin is too little talked about. Apart from the chaos and cost of operating a unified economic system with fifteen autonomous currencies, the stability that a common currency would bring would benefit not simply businesses now operating internationally, but also people whose jobs depend on capital investment in new technology. One of the most serious consequences of British ambivalence on the issue is that these aspects of a common currency are inadequately discussed. That would not be the first occasion on which an important European development had been improperly explained to British voters. (The Thatcher government signed the European Single Act in 1986 without quite knowing what it had undertaken, and then kept silent.) This time, the result may simply be to drift into a situation in which a more fragile sterling remains legal tender, but most trade is carried on in the new European currency. □

Microsoft and the law

The US courts' worries about Microsoft's success should be diverted into more tangible directions.

EVEN Mr Bill Gates, the chairman of the software company Microsoft, should not be surprised that a federal judge has overturned the deal struck between his company and the US Department of Justice at the end of last year. After three years of investigation, the department had concluded that allegations that Microsoft's commercial dealings are designed to stifle competition were partly, but only partly, valid, and the company was required to put up with demeaning external supervision of its commercial contracts for many months ahead. Predictably, Microsoft's competitors were not satisfied with the proposed terms of the deal, and applied to the courts for judicial review. This they will now



get. Whether they will then be satisfied is another matter.

Microsoft is hugely successful. Over fifteen years, it has consistently grown more quickly than any other company in the world. Its annual profits now compare with the British government's annual spending on research. Much of its success derives from a classic recipe — a blend of technical flair and good luck. The flair was the design of the operating system for the first generation of IBM's personal computers, now called PCs. The good luck was IBM's liberal approach, in the early 1980s, to the prospect that its own machines might be replicated by others, or 'cloned'; Microsoft's operating system thus became available to other manufacturers and the company's reputation was thereby enhanced.

Since then, Microsoft has successfully launched a more elaborate operating system for PCs that takes much of the wind out of the sails of Apple Computer's products, still the only distinctive alternatives to PCs on the desktop computer market. Now there is concern that Microsoft's purchase of a company called Intuit will give it a commanding lead in off-the-shelf accountancy software, which the Justice Department is looking at. Others in the United States are alarmed that Microsoft's muscle will give its planned electronic network a commercial advantage over existing competitors as well as giving the company itself a captive market for its software. Meanwhile, the company is developing multimedia products as if it were a publisher, selling intellectual content as well as the means by which a person can read the contents of a storage disk, say. At least so far, there has been no sign that Microsoft has faltered in its pursuit of growth.

What will the courts make of all this? Past precedents suggest that the only certainty is that much time will pass. The inquiry in the 1960s into the old Bell System telephone monopoly, which eventually led to the present patchwork of regional telephone companies and AT&T, took three years to complete. In retrospect, the timing could hardly have been worse-judged. Technical developments in communications have since ensured that the old telephone monopoly would have been quickly eroded in any case. But the tendency for court inquiries into the market dominance of a single company to be overtaken by events is most clearly illustrated by that into the affairs of IBM a decade ago; by the time the courts had finished brooding, IBM was fighting for corporate survival. Judge Stanley Sporkin, the source of last week's ruling, will look foolish if Microsoft goes the same way, perhaps because of the distraction of a long court case.

While Microsoft's competitors' essential complaint is that Microsoft is too successful for their comfort, the judge's problem will be to identify legitimate complaints and then to disentangle complaints of illicit business practices from complaints that the technology of the personal computer is changing too quickly for many people's taste. One condition of the Justice Department's settlement with Microsoft last year is that the company should no longer license computer manufacturers to use its operating systems for periods of longer than one year; that may provide would-be competitors with a chance to steal the company's business, perhaps by designing a better or a cheaper system. But that prospect will not keep Mr Gates awake at night; his company's lead

could be challenged only by huge and speculative investments by competitors not yet extant.

Under US law, it will be a more serious business if the complainants can show that Microsoft has been using its influence as a supplier of operating systems to persuade manufacturers to install its own applications programs on the machines they sell, but even that will not be easily adjudicated. The practice of selling computers complete with a bundle of assorted software is now commonplace, but the terms are rarely as apparent to the ultimate purchasers as they should be. But what if Microsoft evades that issue by making applications programs integral parts of its operating system? That is the real threat. And that is the issue, closely linked with that of the copyright protection software enjoys, on which Judge Sporkin is most likely to stub his toe.

Most software manufacturers are open to complaints of this kind. Everybody knows of simple word-processing programs that have been adapted to function with faster chips and at the same time elaborated, so that they can generate graphics, publish books and even (with grammar-checking 'utilities' as they are called) pretend to write them. The price is accordingly enhanced. For the manufacturers, it is a standard marketing technique, that of selling often unwanted 'added-value' to the captive market of those familiar with the basic program. The high cost of these additions is one of the reasons why proper efforts to protect the copyright of commercial software so often seem rapacious. The protection of copyright would be more easily secured if manufacturers were required to make the extra facilities their programs offer separately available. That, of course, is how Microsoft should be dealt with in the present dispute, but Sporkin should make even its competitors live by the same rules. □

Genetic expectations

The search for the genetic roots of antisocial behaviour is now, and may always, be premature.

Is there a gene 'for' assault and battery, or 'for' serial killing, or for driving motor-cars faster than the speed-limit? These are questions provoked by a conference last week at the Ciba Foundation's London headquarters, at which they were also explicitly discussed. As put, the questions are, of course, nonsensical, largely because there is no reason to suppose that a habitual behaviour can be genetically determined. But, political correctness notwithstanding, there is ample evidence that some aspects of human behaviour (sexual behaviour, for example) are indeed genetically determined. In principle, it is even possible that a tendency to violent behaviour may also have genetic antecedents. Thus there is every reason why the question should be discussed from time to time. But the matter is unlikely to be decided even when there is a complete nucleotide sequence of the human genome; a decision is unlikely to be of much assistance in the defence of modern societies against the prevalence of crime; and this is by no means the most urgent issue in the bearing of genetics on modern life. □

After 20 years, prospects remain bleak for minorities in US science

Atlanta. The American Association for the Advancement of Science (AAAS) chose "Unity in Diversity" as the theme for its 161st annual meeting, which took place over last weekend in the home city of Martin Luther King and his still-elusive version of the American Dream.

But Atlanta is still not united, and US science is not yet diverse. Thirty years after Dr King's speech, blacks have political control of the city's shattered core. But the whites — and most of the wealth — have moved to the suburbs; and the overwhelmingly white gathering of over 5,000 AAAS members reflects 20 years of faltering efforts to make science "look more like America".

Figures recently published by the National Research Council show that in 1993 black Americans were awarded 4.2 per cent of all doctorates in the United States — a figure virtually unchanged since 1978. But the bulk of those doctorates were in education and social science: blacks received just over 2 per cent of life sciences doctorates in 1993, and just 41 physical sciences doctorates out of a total of 3,500. Hispanics have slightly improved their position. But both groups remain underrepresented in the outflow of science PhDs in the United States by a factor of about ten.

"The statistics are very depressing," concedes Francisco Ayala, the president of the AAAS and himself a Spanish immigrant to the US. "Despite all the efforts that have been made, representation remains very low". Although the AAAS board meeting in Atlanta was briefed on the issue, Ayala says, "the fact is we don't know the answers. Perhaps attempts have been made to patch the problem up, when systematic problems need to be approached systematically."

But the vultures are now circling over such efforts as have been made to draw blacks and Hispanics into US science. Programmes to help minority students that exclude whites have been declared illegal (although the ruling has not been enforced). A pending referendum in California could nullify the state's affirmative action programmes, and Senator Phil Gramm (Republican, Texas), a leading contender to be the next US president, has promised to do the same at the federal level — on his first day in office.

Politicians are not the only people pro-

moting such policies. At a recent science policy seminar at the Massachusetts Institute of Technology, James Vincent, chief executive of the biotechnology company Biogen, said that efforts to boost diversity in science are a waste of time. We should, he said, "return to a merit-based system".

James Wyche of Brown University, Rhode Island, who organized a session on racial diversity in the sciences at the AAAS meeting, said that these events were not all bad. "There's been a change in the political and social environment in the United States, and this question is being revisited," he says. "That is not necessarily a bad thing." Some programmes, says Wyche, have been created without an adequate assessment of their effectiveness.

At the session itself, speakers argued that diversity programmes should emphasize excellence, rather than remediation. "I rarely hear of kids from remedial classes doing a PhD," said Freeman Hrabowski, president of the University of Maryland at Baltimore County, whose \$6 million Meyerhoff Scholars' programme, aimed at attracting the best minority students into science and steering them into PhDs, has been widely acclaimed.



Wyche: fears for the future of diversity

But it is at graduate school that the problem intensifies, according to data reported by Clifton Poodry of the National Institutes of Health (NIH). He found that, while 33 per cent of white graduate students complete their PhD, only 14 per cent of minority students do so — a far wider disparity than at any other stage of education.

Poodry says he does not know how much this is due to the relative economic insecurity of minorities. But Hrabowski denies the suggestion that it may be irresponsible to send black students down such a rocky road. "Remember that there are thousands of economically advantaged black people in America, and there are thousands of disadvantaged minorities who are doing well in science," he points out.

The session also acknowledged the crucial requirement of leadership from prominent individuals. Only very recently has such leadership been forthcoming in science. Harold Varmus, the director of NIH, has taken a strong interest in the issue inside the institutes, and was described by Hrabowski as the "godfather" of the Meyerhoff programme.

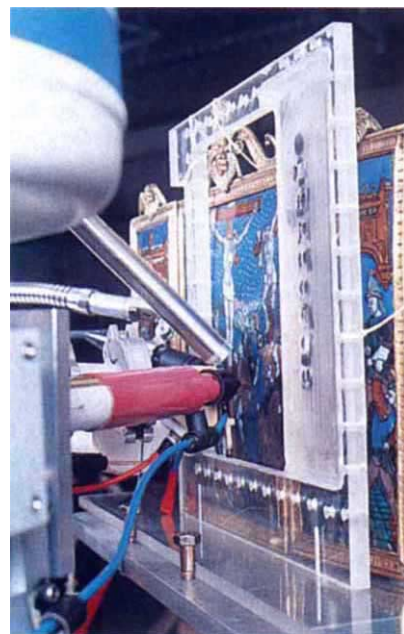
Dan Goldin, administrator of the National Aeronautical and Space Administration ►

Art meets science underneath the Louvre

Paris. **The worlds of science and art collided last week with the opening of the new buildings of the Laboratoire de recherche des musées de France, in the grounds of the Louvre in Paris. Created in 1931, the laboratory has become a centre of excellence for the scientific study of collections, and the restoration of works of art — over 1,000 objects and 430 paintings pass through the laboratory every year.**

The new building is likely to become recognized as a work of art itself. Built at a cost of FF155 million (US\$30 million), the Parisian daylight saturates its 5,000 m² of underground floors — arranged on three levels around a vast central shaft — through a massive glass slab supported by solid glass beams, themselves an architectural first.

Besides the X-ray, infrared and ultraviolet radiation techniques used to unravel the history behind the surface of objects, the centre's 60 researchers are also equipped with a 2 MV electrostatic particle accelerator 'AGLAE'. Its three neutron beams allow the non-destructive chemical analysis



of objects, for example the study of the colours of glass used in the triptyque of "The Crucifixion" (see above), made of enamel on copper in Limoges around AD 1500.

Declan Butler

(NASA) and originally a Republican nominee, has appointed minorities in key positions in NASA for the first time. Addressing the Atlanta meeting, he pledged to involve more "capable women and quality minority scientists" in the work of the notoriously white and male agency.

Yet President Bill Clinton's attempts to appoint a more diverse administration have attracted much derision in the United States, and were almost certainly a factor in last November's elections, when Republicans won almost two-thirds of the white male vote.

Ayala maintains that this derision does not permeate the scientific community. "There is a backlash among the general population, but not among people who practice science," he says. He cites election results to the 11-strong board of the AAAS — which has four women, two blacks, and the Spanish-born Ayala as president — as evidence of members' desire for diversity.

Wyche does not see a backlash on the campuses either. But he fears for the future of racial diversity in science in the United States if the federal government decides to stop pushing for it.

"We have slowly built up support for what we are doing among the majority [white] faculty over the past 20 years. They have supported us because of the protection of the federal government." Without that protection, he fears, that "fragile base of support" will fade away. **Colin Macilwain**

R&D funding 'heading for 25% cut'

Atlanta. Spending by the US government on research and development (R&D) is likely to fall by a quarter over the next five years, according to George Brown, former chairman and now senior Democrat on the newly-named House of Representatives Science Committee.

The veteran California congressman told a AAAS session on the future of the physical sciences that the impact of Republican spending plans on science is hard to gauge. But he added: "my own prediction is for a 25 per cent cut in total R&D spending over the next five years, with some areas suffering even more."

Brown conceded that he had been among those who had encouraged scientists to link their work to "national goals" which had now changed with the Republican takeover of Congress. This, he said, had left the scientific community in "the worst situation"; it was now under fiscal attack, but without the benefit of goals and performance standards, and being judged by "inappropriate measures".

As a result, Brown predicted, basic research will now be worse off than before, isolated by the impending decline in applied research while being subject to new political demands. "Putting on my partisan hat, it is naive to think that research, [whether]

Scientists urged to protest against new 'book burners'

Atlanta. Branding the new leadership of Congress as "book burners", Bruce Babbitt, the Secretary of the Interior, last week called on scientists to rally in defence of three science agencies in his department — the US Geological Survey (USGS), the Bureau of Mines and the National Biological Survey (NBS) — all of which, he predicted, Congress would try to close down before the end of this year.

In a combative address to the AAAS meeting in Atlanta which marked a further hardening of the administration's opposition to Republican plans to cut the budget, Babbitt said that the elimination of the three agencies threatened to "eliminate root-and-branch every trace of science" from his department.

"The proposed destruction of these agencies is the [natural] resource equivalent of book burning," said Babbitt. He also compared proposals to close the USGS in the wake of last year's earthquake in California to "the burning of a few more heretics at the stake" after an earthquake in seventeenth-century Portugal.

But he described the threat to the agencies as "genuine and immediate", predicting that, in the autumn, President Bill Clinton

might be unable to veto a budget bill that would close them down. Furthermore, if Clinton does indeed veto the budget bills, Republicans in Congress have pledged to withdraw all approval for federal spending — even if this means that parts of the government have to close down.

The proposal to close all three agencies is included in a list of cuts prepared by Republican members of the House Budget Committee last April. The list is referred to in the *Contract with America*, the Republican election manifesto, as an example of how savings could be made. But Republicans deny that the specific cuts form part of the *Contract*.

[Robert Walker (Republican, Pennsylvania), chairman of the House Science Committee and vice-chair of the Budget Committee, told a closed meeting of senior scientists in Washington last week that elimination of the USGS was 'off the list' of Republican cuts.]

At the time of going to press, no-one in Walker's office was available to confirm his comments. Bonnie McGregor, deputy director of the survey, said she was unaware of them, and warned against complacency in the run-up to appropriations hearings due to start next week.]

Of the three science agencies at the Interior Department, the most seriously threatened is the NBS, recently and defensively renamed the National Biological Service. But some officials say that Babbitt himself took a risk by setting up the NBS in 1993 as a politically contentious consolidation of several previously obscure research divisions within his department.

The threat to the survival of the NBS has left natural resource sciences such as conservation biology and ecology in disarray. Researchers had been expecting that, after the foundation of the survey, Babbitt would start to provide it with money to fund the collection of badly needed data; officials now concede that this will not happen.

Instead, they expect Congress to attempt to rescind part of the \$167 million already appropriated to NBS for the current year, before moving on to more drastic measures. "I'm just a simple university professor suddenly thrown into the world of Washington," protests H. Ronald Pulliam, the director of the NBS, who happily held a tenured post at the University of Georgia until last summer.

Pulliam says that good science at NBS need not necessarily lead to a new outbreak of regulation that Republicans claim to fear. "The more we know about natural systems, the more chance there is that we can have the economic progress we want, without losing the biological diversity we need," says Pulliam. **Colin Macilwain**

basic or applied, will not be linked to the conservative Republican social and political agenda."

Brown called for new thinking about the scale and scope of the US university system, which he feels may not be sustainable at its current size. "We can no longer deny the excess capacity, or more correctly unfocused capacity, in our higher education system," he said.

He also said that, regardless of recent political changes, the physical sciences are likely to prosper in post-Cold War America only if they addressed the nation's social and need.

"More concretely, if the scientific community cannot reconnect with basic social values, you will find yourselves in a role as central to policy making as a Democrat in Washington — a status I wouldn't wish on anyone," said Brown.

* A week earlier, speaking in Washington, Brown had pointed out that the budget proposals put forward by the administration would take total spending on civilian and defence research and development below one per cent of the gross national product for the first time since 1958. "I am very concerned, but I'm afraid that the situation will only get worse after the new Congress gets through with it," he said. **C. M.**

UK observatories head for a new management shake-up

London. Britain's arrangements for running its ground-based astronomy programmes are being thrown back in the melting pot — only three years after the last upheaval, which led to the Royal Greenwich Observatory, now based in Cambridge, and the Royal Observatory in Edinburgh reporting to a single director.

The new moves could lead to the 'privatization' of Britain's telescopes at La Palma, in the Canary Islands, and on Hawaii, both of which are currently financed and run by the Particle Physics and Astronomy Advisory Board (PPARC). They could also result in further changes in the role of the two British 'observatories' — both of which under one scenario might become incorporated into their neighbouring universities.

PPARC officials emphasize that a range of options is currently under consideration for achieving its main objective, namely a separation between bodies responsible for setting policy and funding research, and those responsible for carrying out the resulting research projects.

But they also claim that the various possible changes now being evaluated will address fundamental structural problems that were inadequately resolved in the last shake-up, carried out by its predecessor, the Science and Engineering Research Council.

The new review has been prompted partly by the findings of a committee chaired by Jim Hough of the University of Hertfordshire into arrangements for the support of optical infrared millimetre astronomy, whose findings were presented to the full council last Thursday (16 February).

The panel gave a general endorsement of the high quality of research currently being funded by PPARC in optical astronomy. It also supported maintaining a 'suite' of ground-based telescopes to complement the new international twin 8-metre Gemini telescopes, currently under construction in Hawaii and Chile.

Both conclusions have been welcomed by PPARC. But there has been less consensus on changes proposed by the panel to increase the effectiveness with which research at the telescopes is organized.

The Hough review itself suggests that both the sites in La Palma and Hawaii should be allowed to operate as autonomous bodies. These would be managed by another organization — suggestions range from an individual university to the newly-autonomous Daresbury and Rutherford Appleton Laboratory — and subsequently apply to PPARC for funding.

The review panel also recommends that the task of preserving Britain's national capability in telescope instrumentation should be made the responsibility of a "UK

astronomy centre".

In responding to the panel's conclusions, the full council said it endorsed the idea inherent in encouraging the telescope sites to operate autonomously by ensuring that accountability and responsibility "should be devolved as far down the operational chain as possible."

It also accepted the need to maintain UK excellence in telescope instrumentation development. But council officials are cautious over the specific proposal for a national centre made by the Hough panel, suggesting that the costs and benefits of such a plan need to be carefully compared to those of other options — which might be transferring such responsibility to an existing



body, or leaving universities to carry out the work on a decentralized basis.

Indeed, the council itself is said to have been split over the major thrust of the changes, some members arguing that, since the present arrangements appear to be working successfully, there does not seem to be a pressing need for change.

In concrete terms, one focus of the debate is whether, if the telescopes are to be 'privatized', then each should apply separately to PPARC for funding — or whether their research strategies should be co-ordinated by a new committee.

Given the many uncertainties that exist, PPARC's decision to make a careful cost-benefit analysis of its options has been widely welcomed. Alec Boksenberg, for example, the director of the Royal Observatories, says he is "pleased that there will be some real in-depth studies of the different options before any decisions are taken."

PPARC itself accepts that many of the costs of a major restructuring — such as those involved in handing over responsibility to a university — need careful assessment. But it hopes to have sufficient calculations completed by its next meeting in May to reduce the "delay and uncertainty" caused by the current debate.

David Dickson

Institute Pasteur to adopt 'automatic' misconduct process

Paris. The Institut Pasteur in Paris has established a new set of administrative procedures which it says it intends to apply "automatically" to all suspected cases of scientific misconduct.

According to internal Pasteur documents, the move has been prompted partly by controversy last year over the handling of a previous relatively minor case of scientific misconduct at the institute (see *Nature* 369, 266; 1994). But the documents also give as a broader reason the "evolution of the general context of research".

Under the new system, allegations of "lack of scientific integrity" will, in the first instance, be assessed by the management of the Institut Pasteur. If it is decided to take the issue further, an investigatory commission will be appointed, made up of three to five members of the institute's scientific board and supplemented by outside scientists if particular expertise is required.

Misconduct is defined as "deliberate fabrication of experiments, presentation of falsified results" or "the theft of other people's data". The commission would interview those accused, as well as other witnesses. If it confirmed that grounds existed to suspect misconduct, then the institute's standard disciplinary procedures would be invoked.

Labour union representatives of staff at the institute have generally welcomed the move, in particular because it adds a preliminary step before the existing disciplinary procedures, which they consider ill-suited, if followed in isolation, for misconduct cases.

In particular, it has been argued that the formal nature of current procedures carries a double jeopardy; management is often reluctant to use them, while those subjected to the procedures risk damage to their reputation, even if subsequently exonerated.

But staff representatives are also critical of the fact that, under the new system, the investigatory commission will be nominated by the Pasteur management. They claim that this opens the possibility of individuals being accused of misconduct (or protected against such charges) on political grounds, and that an independent, elected commission would have been preferable, in that it would provide "absolute transparency".

Maxime Schwartz, the director general of the institute, points out however that one-third of the institute's scientific board, from which the commissions would be drawn, are elected by Pasteur staff, while a further third are scientists from outside the institute.

Other observers say the move reflects a shift at Pasteur away from considering all cases of misconduct as major crimes, leading inevitably to the end of a researcher's career — and also harming the institute's reputation if publicized.

Declan Butler

German minister backs more growth for basic research

Bonn. In his first statement on research policy since taking office last November, Germany's new minister for education and research, Jürgen Rüttgers, has promised that basic research can expect to receive a continued increase in support from the federal government.

In particular, Rüttgers has expressed his backing for a new five-year financing agreement with the Max Planck Gesellschaft (MPG), which runs basic research institutes, and the Deutsche Forschungsgemeinschaft (DFG), which provides grants for basic research projects, mainly in universities.

This agreement, which would run from 1996 to 2000, would succeed the previous 'five-by-five' agreement which has guaranteed both institutions a five per cent budget rise during the first half of the 1990s, at a time when basic research elsewhere in Europe has fallen victim to the economic recession.

Rüttgers, who took over a new ministry combining education and research (BMBF) last November, has put forward his plans in a document published in Bonn earlier this month. In it, he outlines his strategy for providing long-term support for Germany's industrial competitiveness by reforming higher education and providing a strong base in both basic and applied research.

On the question of additional funds for basic research through the DFG and MPG, Rüttgers admits that his ministry cannot do this on its own, as a final decision will require the support not only of the federal finance minister, but also of the Länder, which foot half of the bill for both agencies. But he says that such agreement is essential for the long-term planning of basic research.

Rüttgers is more reserved about the future of Germany's 16 national research centres. While emphasizing that the centres are essential for long-term and interdisciplinary research programmes, he also indicates that he intends to keep pressure on them to serve industry's needs more directly.

Rüttger's main concern is the reduction in research support by industry, whose share of total research funding in Germany fell from 62 per cent in 1989 to 59 per cent in 1993. The most recent figures for last year suggest that this downward trend is continuing, and Rüttgers is keen to help correct the situation.

Under one initiative, for example, the BMBF is to set up a group to suggest how Germany's notoriously bureaucratic licensing procedures, particularly those relating to safety issues, might be modified to create a more research-friendly environment in industry.

The ministry will also be responsible for organizing the activities of a new council for

research, technology and innovation being set up by the chancellor, Helmut Kohl, to advise him on ways in which the scientific community and industry can work together more productively. The members of the council are expected to be named shortly. One of their first topics for discussion is likely to be information technology, a field in which Germany lags internationally.

Rüttgers has also broken with traditional reticence on Germany's nuclear research programme — now confined to decommissioning and general safety issues — by saying that the BMBF should support young scientists working in nuclear energy and reactor technology.



Rüttgers: power to introduce changes

mood turn pronuclear.

Biotechnology will also benefit from Rüttger's regime. The minister says that he strongly supports Germany's increased participation in the human genome programme, and has proposed spending DM50 million (US\$33 million) a year, ten times more than the current funds available through the DFG, for studies in human genetic diseases.

All this will cost money. The 1995 budget, delayed by last autumn's general election, will probably not be approved until April at the earliest. But Rüttgers is confident of obtaining an increase for research close to the 2.7 per cent that he proposed at the end of last year. The statement put out by the new coalition government shortly after the election spoke of "higher than average" financial support for the BMBF.

Rüttgers silence up to now on his policies has been the result of the time taken to merge the education and research ministries. The process — now completed — was far from painless, as many experienced staff lost their posts.

But Rüttgers can now draw higher education into his plans to create an environment to support long-term industrial and cultural needs. He plans to implement changes that have long been debated, and may well have more power to do so than his predecessors through his often scorned, but nonetheless influential, title of "minister for the future".

Alison Abbott

Dutch universities 'need shorter courses for undergraduates'

Munich. The Dutch Scientific Council for Government Policy has come out in support of a government proposal to abandon the 'continental' system of university education of relatively long undergraduate courses in favour of a system with shorter (and cheaper) courses similar to those offered by British universities.

In the Netherlands, as in most continental countries, every student with a high-school leaving certificate has the right to a university place. In practice, this has led to overcrowding of universities and little teacher-student contact. Students tend to take up to six years to complete a degree course, which includes a significant research component.

According to a report published last week, the science council says that a student's right to university education should be defended. But it says that there should be more rigorous selection after the first year of study. At present, most students only sit the first selection examination when they feel adequately prepared for it — which can often be three years after the start of their studies. As a result, few are filtered out at that stage.

The report also argues that the traditional von Humboldt method of education, in which all undergraduate students are given a training in research, is inappropriate nowadays. It suggests that the total length of undergraduate degrees should be shortened to three years by reducing or removing the research component. Students wishing to pursue a career in research would then go on to take a further degree.

But Dutch universities are not happy about abandoning the von Humboldt system. In a report of their own, published a week earlier, they argue that shortening courses would lower standards. Ruut de Moor, the former rector of Brabant University who headed the committee responsible for the report, argues that "this would make us an exception in continental Europe".

The universities agree that the first selection examination for undergraduates should be tougher, but say that students should be allowed two years to prepare for it. The government will now debate the two reports and is expected to make a policy statement in September.

The government has already backed down from its demands for an annual saving of Dfl.500 million in higher education costs by 1998 (see *Nature* 371, 95; 1994). At the end of December, it agreed with the universities to reduce the annual saving to Dfl.200 million, to be achieved in stages by the year 2004. Much of the saving, the government hopes, will come from the proposed shorter study time currently being debated. **A. A.**

NIH resolves dispute on cancer gene patent

Washington. An acrimonious dispute over who should be named on the patent application covering the breast cancer gene *BRCA1* has now been resolved.

Under an agreement announced by the US National Institutes of Health (NIH) last week, two scientists from the National Institute of Environmental Health Sciences (NIEHS) who were omitted from the original patent applications are now listed as co-inventors in new applications to the US Patents and Trademarks Office.

The dispute was between the NIH on the one hand and the biotechnology company Myriad Genetics and the University of Utah on the other. The three groups have agreed to work together to develop diagnostic products as rapidly as possible. Meanwhile, the NIH has agreed to withdraw a counter-application which it filed last November in protest at the original patent filings (see *Nature*, 372, 118; 1994).

The disagreement erupted last September, in the wake of a paper published in *Science* announcing the discovery of the *BRCA1* tumour suppressor gene. Women who inherit a mutated form of the gene have an increased likelihood of developing breast or ovarian cancer; indeed, a study in the *Journal of the American Medical Association* puts the lifetime risk for such women of developing breast cancer at 86 per cent.

Shortly before the *BRCA1* discovery was made public, the University of Utah and Myriad Genetics filed patent applications

covering the nucleotide sequence of the gene. The applications named six co-inventors, three from the university and three from Myriad, but not two NIEHS scientists — Roger Wiseman and Andrew Futreal — who had worked on part of the gene.

If the patent had been granted in this form, the NIH would not have been entitled to receive any revenue from diagnostic kits or therapies based on the identification of the gene, even though it had both supported the research at NIEHS and contributed to the cost of the work carried out at the University of Utah.

According to Richard Koehn, vice president for research at the University of Utah, the two NIEHS scientists were excluded from the original patent application in order to prevent any third-party challenge on the grounds that the two should not have been named as co-inventors.

Koehn says such an argument could have been made because the two only worked only on parts of the gene, and could not have independently known the full significance of the sequences they were studying. "You can't simply designate someone an inventor", says Koehn, pointing out that the six people named in the original patent applications had access to the whole

sequence of the gene.

But an NIH official says that the original exclusion of its scientists could have been a result of a misunderstanding of patent law, pointing out that the scientists inevitably knew what they were working on as a consequence of their collaboration with the Utah groups.

Wiseman's and Futreal's names now appear on those parts of the patent application dealing with the sequence they worked on, and the NIH is entitled to revenue from any commercial product stemming from the patents.

At the same time, the three parties have agreed that exclusive, worldwide commercial rights to the gene are retained by Myriad and its licensees, Eli Lilly and Co. and Hybritech Inc.

Disagreements about who can legitimately be named on a patent are not unusual, according to Kate Murashige, a patent attorney in Washington DC. Only a few months ago, for example, the NIH lost a similar case against the pharmaceutical company Burroughs Wellcome. Last week's announcement of agreement between the NIH, Myriad and the University of Utah should forestall legal action between them.

Helen Gavaghan

More focus urged for Japan's drug research

London. Japanese companies need to apply a greater focus to their research and development efforts if they are to compete more effectively with their US and European counterparts, according to an analysis of the global pharmaceutical industry published in London last week by the management consultancy Datamonitor.

An analysis of the relative competitiveness of pharmaceutical companies by region showed that, between 1989 and 1993, the average compound annual growth rate for Japanese companies was only four per cent. This compared to 11 per cent for European companies, and 12 per cent for those in North America.

In Japan, says that report, expenditure on research and development has been lower than elsewhere, with the result that "there is a general lack of new innovative products coming out of most Japanese companies". But the report adds that the picture is now starting to change, with overseas expansion becoming a necessity, and research being targeted more effectively. □

Medical schools fight their corner

Washington. The leaders of more than a hundred US medical schools last week appealed to Congress not to make cuts in two important sources of financing: the reimbursement of costs associated with federally funded research, and a 'medical education' adjustment made on federal payments for care of the elderly.

The academics visited members of both houses — including Newt Gingrich, speaker of the House of Representatives — as part of a massive lobbying effort in support of academic medicine. In particular, they defended the research and teaching that make medical treatment at academic centres more expensive than elsewhere.

At a series of events coordinated by the Association of American Medical Colleges, the visiting medical leaders claimed that social and political changes are combining inadvertently to threaten research and teaching. "You can only take so many hits before the wagon goes in the ditch, and we're about ready," said Jeffrey Houpt, dean of Emory University school of medicine.

As observers point out, the problem has been growing for a number of years. Indeed Judith Vaidukaitis, former head of the clinical centre at the National Institutes of Health, and now director of the NIH's National Center for Research Resources, says although the medical centres are "get-

ting killed", they "should have seen the truck coming a long time ago".

Several factors contribute to the current problem. One is that the large teaching hospitals treat about half of the indigent patients in the United States — including many individuals who are uninsured, but do not qualify for federally funded Medicaid.

Second, say the medical school heads, the additional costs of teaching and research, combined with the fact that the academic centres treat more complex cases, makes the medicine they practise more expensive than in a non-teaching hospital.

Furthermore, individual states are finding it hard to maintain contributions for indigent patients, while the federal government is contemplating cuts in its programmes for elderly or uninsured patients. And newly formed medical groups offering managed care are demanding reductions in the price of services they contract from the big teaching hospitals.

These factors, say medical centre deans, pose a threat to research and teaching just at a time when modern biomedical research is providing exciting leads for clinical research. "If something is not done now, someone will run for president in 10 years time on the basis of reinvigorating American medical colleges," says Houpt.

Helen Gavaghan

France takes control of genome programme

Paris. The French government is planning to take over direct control of publicly funded research on the human genome from its national genome agency, as part of a wider effort to develop a national strategy for the life sciences.

Under plans due to be announced next week, funding for the agency, known as the Groupement de Recherches et d'Etudes sur les Génomes (GREG), will this year be reduced from FF80 million (US\$5.4 million) to FF20 million. Rather than supporting all genome research, the GREG will in future be restricted to funding work on model animals and plants.

The money taken from the GREG's budget will be given to the ministry of higher education and research to create new programmes on genetic function, medical genetics, and genetics and the environment.

Technician charged after CERN sabotage

Paris. A technician at the European Laboratory for Particle Physics (CERN), which straddles the border between France and Switzerland close to Geneva, has been charged with theft and extortion after the discovery that two accelerators at the centre had been sabotaged.

Early in the morning of 13 February, Nicolas Blazianu — who has worked at CERN for 27 years — cut cables and removed 1,200 electrical components needed for the running of the proton/synchrotron (PS) accelerator and the PS booster (PSB). Both devices are used to accelerate electrons and positrons before these are introduced into CERN's large electron-positron collider (LEP).

The technician later called a member of CERN's management to demand a ransom of SFr2 million (US\$1.5 million) and the dismissal of his ex-wife, an administrator at the laboratory. But he eventually gave himself up, and has since provided CERN with information on the location of the components, which he had meticulously hidden around the CERN site.

It is not yet clear whether it will be possible for the accelerators, which had been shut down for maintenance in December, to be restarted in April as had been planned. Even if the components have not been damaged, the accelerators will need to be recalibrated and their beams retuned before being put back in operation.

Following the charges against Blazianu, CERN has itself launched an internal inquiry into the incident and its consequences for the running schedule of the site. The ease with which the centre's machines were sabotaged is likely to result in a review of internal security procedures. **D.B.**

Each will be run by an expert committee set up within the science ministry.

Claude Griscelli — an adviser to Francois Fillon, the science minister, and considered to be the main architect of the planned national strategy for life sciences — claims that the ministry is well placed to "coordinate" the genome research carried out by the various research organizations.

Indeed, this is the philosophy behind the national strategy for life sciences itself, which will group all biological research carried out by the Centre National de la Recherche Scientifique (CNRS), the Institut National pour la Santé et Recherche Médicale (INSERM), and other research organizations within 14 "horizontal" themes, each run by an expert committee. Details of the themes have yet to be announced.

But the planned changes in the organization of genome research are being strongly opposed by Piotr Slonimski, the director of GREG. He argues that it will not provide any extra money for such research, but will merely introduce new layers of bureaucracy which could, he claims, delay procedures for approving and financing grants; at present, he says, the GREG pays out grants within two weeks of approving a research proposal.

Slonimski also points out that, even though GREG will receive FF20 million this year, the agency has only FF8 million left to launch new programmes, claiming that the ministry wants to abolish GREG.

GREG's scientific board decided last week to evaluate all the research proposals it has in hand. Those that fall within its new restricted jurisdiction will be financed; its evaluations of the rest will be forwarded to the ministry "to take into account or not, as it sees fit".

Some claim that the change has been prompted by a feeling in both scientific and political circles that the policies of the

GREG have not been sufficiently ambitious. But they are reluctant to criticize Slonimski, arguing that he should be given credit both as an eminent geneticist, and for having brought order to France's fragmented genome efforts.

Slonimski accepts that his support for academic laboratories has been eclipsed by the success of the industrial-scale approach to genome sequencing and related research pursued by the Généthon laboratory outside Paris. But he argues that the two strategies have not been incompatible.

Indeed, Slonimski points out that although GREG has spent most of its funds on research carried out by Généthon, he has felt it important to fund laboratories working in other areas as well. The new plan, he complains, will separate responsibility for research on the human genome from studies of the genomes of other model organisms.

One CNRS official acknowledges that the ministry has been motivated by a desire to take control of human genome research out of GREG's hands. But, he argues, fears that this would lead to genome research becoming "more medical and applied" have not been borne out. "CNRS labs will be able to get funding [for genome research] without compromising their mission [to do basic research]," he says.

The official also defends the ministry's decision to take over responsibility for bioinformatics from GREG, arguing that this is a generic tool that needs to be coordinated across all biological research.

Few details of the ministry's planned national strategy for the life sciences are yet available. But some feel that these are likely to be less ambitious than initially expected; plans for interministerial collaboration for example, have fallen through because of disagreement over territorial responsibility.

Declan Butler

Indian confirms identity of plague

New Delhi. An independent committee set up by the Indian government has confirmed that the disease that broke out in Surat four months ago was indeed plague — refuting earlier reports that some other cause might have been responsible (see *Nature*, 371, 547; 1994).

The committee's interim report endorses the earlier findings of a team from the World Health Organisation (WHO) and finally puts to rest the controversy triggered by various Indian microbiologists — including N. P. Gupta, a former director of the National Institute of Virology — and based on what were described at the time by some scientists as various "baffling and mysterious" aspects of the outbreak.

The committee was made up of 11 lead-

ing microbiologists and epidemiologists, and was formed immediately after the departure of the WHO team. It claims to have isolated and grown the causative agent and identified it as *Yersinia pestis* after a battery of biochemical tests (the WHO team relied on circumstantial and sero-epidemiological data). Final confirmation came from PCR analysis of DNA extracted from the isolates and autopsy specimens.

Meanwhile the government is considering a \$30-million proposal to establish a national disease surveillance network which would provide warning of any local outbreak of a disease before it becomes an epidemic. The network would be run by the National Institute of Communicable Diseases in New Delhi. **K. S. Jayaraman**

ANC adviser takes over at science ministry

Cape Town. Roger Jardine, for the past three years the leading science policy adviser to the African National Congress (ANC), has been appointed director-general of South Africa's Department of Arts, Culture, Science and Technology, the ministry responsible for the government-funded research councils.

A radiological physicist by training, Jardine is at 29 the youngest person ever to head a government department in South Africa. But he faces the formidable task of directing a ministry beset by both political problems and decreasing funds.

Jardine says that the government plans to carry out a review of the activities of each of its seven science councils, as well as the National Accelerator Centre, the focus of recent controversies about government support for nuclear research (see *Nature* 365, 776; 1993). The review will take place over the next 18 months in conjunction with a research foresight study loosely modelled on Britain's technology foresight exercise.

But Jardine acknowledges that the most pressing problem facing South African science is a lack of funds. The science vote has been reduced by 27 per cent in real terms since 1987, and is likely to drop another four per cent in next year's budget.

Jardine left Johannesburg in 1986 to study at Haverford College in Pennsylvania, before completing a master's degree at Wayne State University in Michigan. He

returned to South Africa in 1992 as co-ordinator of science and technology policy in the ANC's department of economic planning.

His new post will give him an opportunity to help put into practice some of the ideas developed previously within the ANC, eventually leading to a restructuring of the South African science system. But he faces an uphill task. Although the minister responsible for Jardine's department, Ben Ngubane, has protested over the decline in funding for research and development, he appears to lack the influence within the cabinet to resist the cuts.

Furthermore, despite the new government's commitment to transparency, Ngubane has declined to make public the recommendations of his advisory committee on the allocation of the vote among the councils for the next financial year. The former government's Scientific Advisory Committee was heavily criticized for keeping its recommendations to the government secret (see *Nature* 362, 384; 1993).

Ngubane announced at a press conference earlier this week that a National Council for Science and Technology will be established by June. It will replace the advisory committee, which was intended only as

an interim body, and the procedure by which it is to make its recommendations has yet to be determined.

In terms of special initiatives, Jardine says there are two priorities for South African research and development: finding new ways of using technology to boost economic development and of enhancing the standard of living in the rural areas.

He must first organize his new department, which has been unable to run efficiently owing to lack of staff. It has inherited some officials from the former Department of National Education, and others from the Department of Trade and Industry. But 62 posts, including almost three-quarters of top management positions, remain vacant.

But Jardine's most unenviable task is to address the department's political difficulties. The deputy minister, Mrs Winnie Mandela, appears to have escaped being fired by her estranged husband, Nelson Mandela, last week, following the resignation of 11 members of the executive committee of the ANC Women's League, which she heads. If she rides out this particular political storm, Jardine's most difficult challenge will be to guide her and Ngubane along a common path.

Michael Cherry



Jardine: facing tight funding and political challenges

Australian researchers hold government to its promises

Sydney. Biomedical researchers are putting pressure on the Australian government to honour an election promise made by the prime minister, Paul Keating, in 1993 that he would increase research grants by an additional \$A200 million a year.

Scientific bodies, frustrated by lack of action on the promise made during a hard-fought election campaign, have been lobbying government ministers through reports, meetings and briefings on the state of biomedical research.

So far, however, the lobbying has been kept deliberately low-key. "We were told it might well be counter-productive to lobby too hard," said Fiona Stanley, director of the Institute of Child Health Research at the Princess Margaret Hospital for Children in Perth, Western Australia.

"We were told that the people in Canberra considered researchers to be greedy, ungrateful people who would just keep on asking for more money," said Stanley, who is convenor of an independent working group of researchers that prepared a report on the issue and briefed Keating two months ago.

Whatever the view held by federal politicians of medical researchers, Stanley and

other biomedical scientists believe strongly that the government should meet an election promise to boost spending on health research to 2 per cent of the total health budget by the year 2000.

The precise target is still being debated. But biomedical scientists understood the promise to be based on statistics produced by the Australian Institute of Health and Welfare, which showed that current spending on research was around 1.4 per cent of the total. So far, the government has increased funds distributed by the National Medical and Health Research Council (NMHRC) by only a token amount.

Ian McCloskey, director of the Prince of Wales Medical Research Institute in Sydney, New South Wales, says researchers fear that, if the government does not increase research funds in the next federal budget, due to be decided over the next few months, they are unlikely to see any additional funds.

But the costs will be high. Stella Clark, president of the Australian Society for Medical Research (ASMR), which represents 9,000 researchers, says that in order to honour its promise, the government needs to add an extra \$A40 million a year to the

NMHRC's research budget for each of the next five years.

In a report recently prepared by the ASMR, and submitted to the Australian cabinet, the society says that institutional support for health and medical research and development is in long-term decline at both federal and state levels, the first affecting universities and the second hospitals.

It also claims that, in comparison to other advanced countries, Australia has a "weak track record" for individual philanthropy and corporate support of research in the public interest. As a result, many Australian researchers have been living on short-term grants of between one and three years, and have had to spend too much time seeking support for jobs that remain badly paid.

Early in 1994, Senator Graham Richardson, the former minister for health and a powerful figure in Australia's Labor government, claimed that the required extra funding being sought by the biomedical community had been locked in place. But since his retirement nothing has happened, despite a declaration of support for the funding increase from the present health minister, Carmen Lawrence. **Mark Lawson**

Misinterpreting Aquinas

SIR — John Godfrey's arguments¹ in his not-so-veiled attempt to justify human embryo experimentation rest on fallacious reasoning and citations made out of context.

While accepting Godfrey's claims about the teaching of St Thomas Aquinas as agreeing with Aquinas's thought, we sense that a false impression is created through his convenient omissions of what Aquinas also says. Aquinas did *not* believe in continuous, gradual development. Rather, he held that during ontogenesis the embryo takes on a succession of discrete forms, the process being accomplished not by any *internal* process, but by the *external* power of the seed (*a virtutis quae est in semine*, *Summa Theologica* IaQ.118a.1ad4). Aquinas does specify the number of days this process requires; however, he categorically considers abortion (and, by extension, human embryo experimentation) as evil.

We also take issue with Godfrey's expedient deification of Aquinas. For although he "continues . . . to be the master of philosophical and theological universalism", Aquinas was subject to the same fallibility and corruption as the rest of us. Thus his thought is considered an extraordinary help, not the gospel truth.

Finally, we fail to see why a theology of person must be conceived as a transliteration of embryology's current grasp of the early stages of human development, as Godfrey suggests. Although it is true that embryology and modern genetics illuminate biological aspects of human reproduction and development, providing valuable knowledge for practical and humanitarian endeavours, these sciences alone inform us only in part about what it is to be a person. It seems to us that the essential meaning of an individual human life can be apprehended through modes of thinking commonly considered philosophical or theological. Clearly, one's assessment of the irreducible worth of a person influences the significance one attaches to information suggesting when during embryogenesis a human being may rightfully be called a person.

David A. Jones

Blackfriars, Oxford, UK

Donald T. Haynie

New Chemistry Laboratory,

University of Oxford,

South Parks Road, Oxford OX1 3QT, UK

SIR — The axiom "There is no moment when human life starts" must be discarded as unsound. If it denotes that life is continuous from one generation to the next, then it is a boring truism: spontaneous generation theories were refuted long ago. If used to convey the idea that (human) life appears as a diffuse living

magma, from which individuals of obscure inception emerge, it is obscurantism.

Godfrey¹ concludes, from the fact that both egg and sperm are alive, that there is not a clear-cut beginning for individual human life. That seems an unfair inference. Fairness forces us to acknowledge that egg and sperm differ utterly from the zygote. Gametes are wonderfully differentiated but terminal cells, fatefully condemned to die in a few hours or days. Fertilization changes things radically: besides a complex cellular process, it originates a new, sudden and violent burst of life that grows and lasts years and years.

When the Pope speaks of fertilization, he is not analysing cellular or molecular phenomena in a reductionistic mood. He is dealing with the human action of begetting children. Godfrey's blurred biological picture of fertilization denies a fundamental fact of life: fathering. If human life does not begin with fertilization, what is then the biological and human decisive role of the father? Children would appear then as products of an odd and nameless asexual continuum, not the conception of the love and flesh of a woman and a man.

As scientists, we must not cut ourselves off from the real world. Godfrey's Commentary, with its adversarial feelings towards John Paul II, represents, among other things, an impoverished and unnatural version of human fertilization.

Gonzalo Herranz

University of Navarra,
School of Medicine,
31080 Pamplona, Spain

SIR — Godfrey argues that the Pope ignores most of modern genetics and embryology¹. But he bases his criticism on a misunderstanding of what the Pope and the Roman Catholic Church believe.

Details of the time taken for fertilization and development of the nervous system are not central to the concept of the person as understood by the Church. Even so, the advances in our understanding of fertilization and embryology have emphasized the importance of fertilization as the start of the life of a new individual.

The presentation of St Thomas Aquinas's view of ensoulment is a most remarkable adaptation of what he actually said. Examination of articles XXVII and XXXIII of his *Summa Theologica* clearly shows that he argued that infusion of the soul occurred at the same time as formation of the body, with no distinction between the sexes. Subsequently, 38 years after his death, the Council of Vienna formally defined that the soul is the 'form' of the body. ". . . Spirit and matter in man are not two natures united, but rather their union form a single nature."² The

dignity of Man is dependent on this single nature and it explains why Catholics give equal respect to all humans regardless of their state of development or intellectual ability.

It is a sad reflection of our times that there are many who, though professing the Catholic faith, ignore its teaching. The Pope has addressed this problem in his recent encyclical *Veritatis splendor*³. Catholics believe that there are such things as absolute truths that cannot be changed — otherwise they would not be truths. God has given us free will and so we have the freedom to accept or reject the truth; we cannot change it. Aquinas was right in arguing that fairness and common sense should be used in applying human laws. But the teaching of the Church in the area of reproduction is based on natural law. Just as a scientist cannot change the law of gravity because it does not suit his purpose, so too the Church cannot change the nature of Man.

Many have used the fear of overpopulation to criticize the Church's stand against artificial methods of contraception. Those interested should refer to data detailing the efficacy of natural methods⁴. Those data and the subsequent correspondence⁵ prompted the editor of the *British Medical Journal* to comment: "This correspondence, I must say, has changed what may have been a prejudice on my part against natural methods of contraception."⁶

Michael Jarmulowicz

(Hon. Secretary to the
Guild of Catholic Doctors)
6 St Andrews Road,
London NW10 2QS, UK

1. Godfrey, J. *Nature* **373**, 100 (1995).

2. *Catechism of the Catholic Church* (Chapman, London, 1994).

3. *The Splendour of Truth Shines*. Pope John Paul II. (Publ. Libreria Editrice Vaticana, 1993).

4. Ryder, R. E. J. *Br. med. J.* **307**, 723–726 (1993).

5. *Br. med. J.* **307**, 1357–1360 (1993).

6. *Br. med. J.* **307**, 1292 (1993).

Tree of life

SIR — "Living organisms range in size from a diameter of about one hundredth that of a typical human cell to a length of 30 metres, with weights ranging from the infinitesimal to that of a medium-sized airliner" — H. C. Bennett-Clark's review of *Diatoms to Dinosaurs* (*Nature* **372**, 629; 1994).

Perhaps because Bennett-Clark is a zoologist he has overlooked the fact that there is another kingdom of living things called plants, which include the largest of all living things, the sequoia trees of California, estimated to weigh more than 1,000 tons. Some airliner!

Harry Miller

3A, Satyanarayanan Avenue,

Boat Club Road,

Madras, Tamil Nadu, India 600 028

Atom microscopy comes of age

Four years after the first demonstration that beams of atoms can interfere with each other just as if they were beams of light, it has been possible to extract useful information from an experiment. But emulators will be few.

QUANTUM duality goes back a long way, to the beginnings of optical interferometers. Of course, in the mid-nineteenth century, there was no reason to believe (Newton's corpuscular theory of optics notwithstanding) that there were *particles* of radiation, or that they might have the properties of waves and of particles in different circumstances. People could work with the interferometers of the late nineteenth century in complete contentment that wave theories could account for everything.

Only Planck's version of the quantum theory rescued the notion that there may be quanta of radiation; in 1905, Einstein's theory of the photoelectric effect clinched the point. Proof of the quantum duality of electrons had to wait a further twenty years. Since then, people's ambitions have enormously expanded. Beams of neutrons are routinely used in studies of the atomic structures of solids, for they too have the properties of waves as well as of particles, with a wavelength (λ) nicely given by the de Broglie specification of $h/(mv)$, where h is Planck's constant and mv the momentum of each particle.

So why not use other kinds of particle instead, thereby further extending the range of wavelengths over which interference or diffraction techniques can usefully be practised? There are some obvious snags. For one thing, charged particles will not do; imagine trying to make beams of slow-moving protons whose electric charges would not blow them apart! (But fast-moving protons may well have quantum wavelengths comparable with nuclear dimensions and so be probes of the internal structure of atomic nuclei — as well as of their own.) Another is that the electronic structure of an atom may interact with other objects in its path, perhaps making the collision inelastic.

None of that has deterred people from the construction of instruments dependent on the wave properties of neutral atoms. The first demonstrations that the matter-waves of atoms will indeed interfere with each other were independently provided just four years ago, by a group from the University of Konstanz (O. Carnal & J. Mlynek *Phys. Rev. Lett.* **66**, 2689–2692; 1991) and another from the Massachusetts Institute of Technology (MIT) (D. W. Keith, C. R. Ekstrom, Q. A. Turchette & D. E. Pritchard *Phys. Rev. Lett.* **66**, 2693–2696; 1991). The former used beams of metastable helium atoms, the latter went for sodium atoms instead.

The difficulty of these experiments is constructing the slits and gratings through which the atoms must pass, and which have dimensions measured in fractions of a micrometre.

A team of six grown from the MIT group (two of whom are also affiliated with the University of Innsbruck) has now neatly turned one of the snags of atom-based interferometers — the uncertainties caused by collisions — to their advantage (J. Schmiedmayer *et al. Phys. Rev. Lett.* **74**, 1043–1047; 1995). Since next to nothing is directly known of the low-energy collision of one atom with another, why not use an atom interferometer to measure them?

The basic instrument is an elaboration of that described four years ago. There is a nearly mono-energetic beam of sodium atoms, obtained by ejecting through a nozzle into a vacuum chamber sodium atoms carried by argon gas. From the estimated velocity of the gas through the nozzle, the de Broglie wavelength of the sodium atoms is 16 picometres, or 0.16 ångström. The group uses a grating to split the beam into several sub-components, of which only two are needed. The gratings have a geometrical period of 200 nm (a fifth of a micrometre) and are fabricated as films of silicon nitride (Si_3N_4) using all the razzmatazz of silicon-chip technology.

In the original experiment, when the MIT group was simply concerned to demonstrate that interference happens, it operated in as good a vacuum as it could make. Even so, there were bizarre complications. Movement of the equipment as a whole, for example, creates a relativistically significant displacement of the measuring equipment relative to the sodium atoms in the beam, for which reason there is a system controlled by a laser driven servomechanism to correct for displacements of the equipment.

And the equivalent of the photographic film or the photomultipliers with which optical interference would be recorded? Simply a wire heated to the point at which sodium atoms will be ionized and a device for recording the arrival of electrons. Given the system for compensating for the chance movement of the equipment as a whole, it is easier to scan across the diffraction fringes by displacing the equipment than by moving the detector.

That was four years ago. How, in 1995, to cause one of two beams of sodium atoms to interact with the atoms of an

ambient gas when sodium atoms, unlike neutrons, will not pass through the thinnest barriers? Schmiedmayer *et al.* have devised a leaky gas chamber through which to pass one of the two beams of a double-beam interferometer. They have built a rectangular box, open at the ends, which is divided in two by a thin sheet of aluminized mylar foil. One half of the box is fitted with a nozzle through which gas can enter. Except for a gap 200 μm across, the ends of that half of the box are closed by metal baffles.

So the complete experiment consists of three 200-nm diffraction gratings, one to split the beam, one to select the sub-beams immediately in advance of the doubled interaction chamber and one immediately before the hot wire. The gases used to interact with one of the two beams are the noble gases (up to xenon) together with N_2 , NH_3 , CO_2 and H_2O . The essential measurement is that of the phase shift between the two beams occasioned by the presence of scattering gas in one of the two arms. Direct measurement of the density of gas in the interacting cell is evidently not possible, especially because the supposedly empty half of the chamber must become contaminated, but the attenuation of the beams serves as a control on the amount of scattering material.

In reality, the MIT group has been able to finesse even that difficulty by persuading itself that its real interest lies in the ratio of the real and imaginary parts of the forward scattering amplitude — the chance that a sodium atom will be unaffected by an encounter with an ambient atom of the scattered gas. That is directly related to the refractive index of the gas concerned for matter waves of sodium (with a velocity of $1,000 \text{ m s}^{-1}$).

For what it is worth, the results say something about the interaction of sodium atoms with the rare gases. There is an attractive potential-well for each of them, but it is predictably deepest for xenon and decreases steadily from there down to helium. Xenon interacts with sodium as if by a van der Waals interaction, but helium may just as well behave like a hard-sphere billiard ball. The authors say that, apart from improving the sensitivity of their measurements, they hope to extract further information about these scattering processes by varying the energy (and de Broglie wavelength) of their sodium atoms. Good luck to them! will be the general reaction.

John Maddox

Europa's oxygen atmosphere

Donald M. Hunten

THE discovery of gaseous oxygen on Europa, reported on page 677 of this issue by Hall *et al.*¹, is the climax of a 23-year search for this gas on the three outer galilean satellites of Jupiter: Europa, Ganymede and Callisto. But the gas pressure is low, a mere 10^{-5} microbar.

In June 1972 Ganymede, the largest moon in the Solar System, occulted an obscure 8th magnitude star. Successful observations were obtained from Java and India; the shadow just missed Darwin,

with the value deduced from the stellar occultation data. They pointed out that their model would apply equally well to any large jovian satellite with exposed ice, specifically Europa and Callisto.

Broadfoot⁴ was therefore encouraged to use the ultraviolet spectrometer on Voyager 1 to observe another stellar occultation by Ganymede, in a spectral region especially sensitive to O₂. Nothing was detected, and the upper limit obtained for the surface pressure was 10^{-5}

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Europa — a moon with atmosphere.

Australia². The diameter obtained (5,270 km) was within 10 km of that found later by the Voyager spacecraft, and there was marginal evidence for an atmosphere with a surface pressure around a microbar.

A few years later, in preparation for the encounters of the Voyagers with the Jupiter system, Yung and McElroy³ proposed that the gas might be O₂. The presence of water ice on the surface implies that the vapour is in the atmosphere at some very low pressure. It is rapidly dissociated by ultraviolet radiation from the Sun, and the hydrogen escapes, leaving the oxygen to build up until it is in steady state with its loss processes. The most important of these processes follows photolysis of O₂ near the top of the atmosphere by energetic solar photons (wavelength less than 202 nm). If a photon possesses more energy than is required to dissociate a molecule, the excess can appear as kinetic energy, shared equally between the two oxygen atoms. The one that is directed upward can escape if its energy exceeds 0.63 electronvolt. Yung and McElroy found that the surface pressure could be as large as 0.4 to 1 microbar, in agreement

with the value deduced from the stellar occultation data. They pointed out that their model would apply equally well to any large jovian satellite with exposed ice, specifically Europa and Callisto.

The answer was pointed out a few years later in a review by Kumar and Hunten⁵. There are two possible states for such an atmosphere, differing in O₂ pressure by the remarkably high factor of 10^5 ; Yung and McElroy discussed only the high-pressure one, which seemed to describe the result from the 1972 occultation. In this state, the O₂ builds up to a level that partially shields the water vapour from photolysis, reducing the rate to a value that can be matched by the limited loss rate of oxygen atoms (the 'Urey self-shielding mechanism'). In the low state, the photolysis rate of water vapour is limited simply by the amount in the atmosphere, which in turn is controlled by the vapour pressure and any additional sources such as bombardment by ions from the jovian magnetosphere. In principle, small changes in the supply rate could switch a given moon from one state to the other, but the transition would take around 100,000 years.

The Europa observations¹ were made in the far ultraviolet with the newly repaired Goddard High Resolution Spectrograph on the Hubble Space Telescope. They show two spectral features of atomic oxygen in roughly equal intensities: the resonance triplet centred at 130 nm and the spin-forbidden line at 136 nm. Although the optical transition probability of the second line is small, both excited states are easily reached by impact of electrons on either oxygen atoms or O₂ molecules; the latter process involves simultaneous dissociation and excitation. The analysis by Hall *et al.*¹ shows convincingly that both the spectral features, at the intensities observed, can be accounted for by the O₂ source, although there could be a small contribution from oxygen atoms. The flux of electrons of energies in the range of a few tens of electronvolts is taken from the plasma experiment on Voyager. Because these electrons penetrate right through the atmosphere to the surface, the intensities give directly the number of O₂ molecules per unit area.

Remarkably, O₂ was also found just last year on Ganymede, but there it is trapped in the ice rather than being in the atmosphere⁶. Weak, broad absorptions at 577 and 628 nm have now been identified with transitions of two coupled O₂ molecules, each from the ground state to the first excited electronic state. The band at shorter wavelength is produced by a transition to an excited vibrational level of the upper state. Weak absorptions of this type have long been known in the Earth's atmosphere, and they are much stronger in liquid and solid oxygen. On Ganymede it appears that the O₂ molecules are trapped in the water ice, where they may be generated by charged-particle bombardment.

The half-dozen large satellites in the outer Solar System are worlds in themselves, most of them larger than Pluto and even Mercury. All but Ganymede and Callisto are now known to have atmospheres, and these two, like Europa, must surely have some O₂. Titan has a dense nitrogen atmosphere containing some methane; Triton and Pluto have tenuous nitrogen atmospheres and surfaces covered with nitrogen ice; and volcanic Io has drifts of sulphur dioxide ice and wisps of the same gas in its atmosphere. □

Donald M. Hunten is at the Lunar and Planetary Laboratory, The University of Arizona, Tucson, Arizona 85721, USA.

- Hall, D. T., Strobel, D. F., Feldman, P. D., McGrath, M. A. & Weaver, H. A. *Nature* **373**, 677–679 (1995).
- Carlson, R. W. *et al.* *Science* **182**, 53–55 (1973).
- Yung, Y. L. & McElroy, M. B. *Icarus* **30**, 97–103 (1977).
- Broadfoot, A. L. *et al.* *Science* **204**, 979–982 (1979).
- Kumar, S. & Hunten, D. M. in *Satellites of Jupiter* (ed. Morrison, D.) 782–806 (Univ. Arizona Press, Tucson, 1982).
- Calvin, W. M. & Spencer, J. R. *Bull. Am. astr. Soc.* **26**, 1159 (1994).

Two heads are better than one

Bruce J. Schnapp

Two studies reported in this issue of *Nature*^{1,2} show that the unique ability of single kinesin motors to move processively on microtubules for long distances^{3,4} depends on cooperative interactions between the two mechanochemical heads of the native protein. The article by Gilbert *et al.*¹ also provides definitive evidence that the kinesin mechanochemical cycle is fundamentally different from that of myosin, reflecting the distinct *in vivo* functions of these two motors.

Both myosin and kinesin, and their many relatives, are molecular motors that drive various types of motile processes inside the cell by coupling an ATP hydrolytic cycle to a mechanical cycle in which the protein steps along regularly spaced binding sites on a cytoskeletal polymer (actin or microtubules, respectively)^{5,6}. There are, however, important differences in the operation of kinesin and myosin. Skeletal muscle myosin, for all but a small fraction (less than five per cent) of the total cycle time (about 50 ms), is dissociated from or bound only weakly to actin⁷. This prolonged 'off time' makes it technically difficult to measure displacements driven by single myosin molecules *in vitro* because the myosin and actin filaments diffuse away from each other after one step^{6,8,9}. Yet this property suits the *in vivo* function of myosin in muscle contraction, because billions of myosins assemble into the thick filaments of the sarcomere, thus avoiding the diffusion problem encountered with single molecule assays. Because the strongly bound, actively pulling state is brief, like an impulse, power strokes of individual myosins sum to create a large continuous force across each muscle fibril.

By contrast, kinesin is thought to be a vesicle motor, and on the surface of a 100-nm vesicle there is space for only one or two kinesin molecules to attach to a microtubule. Yet remarkably, even a single kinesin will walk processively for long distances while poised over a single microtubule protofilament^{10,11}. This behaviour is remarkable because the intracellular cargo is small (that is, at low Reynolds number) and therefore experiences large-scale (micrometres per second) thermal motion, which would

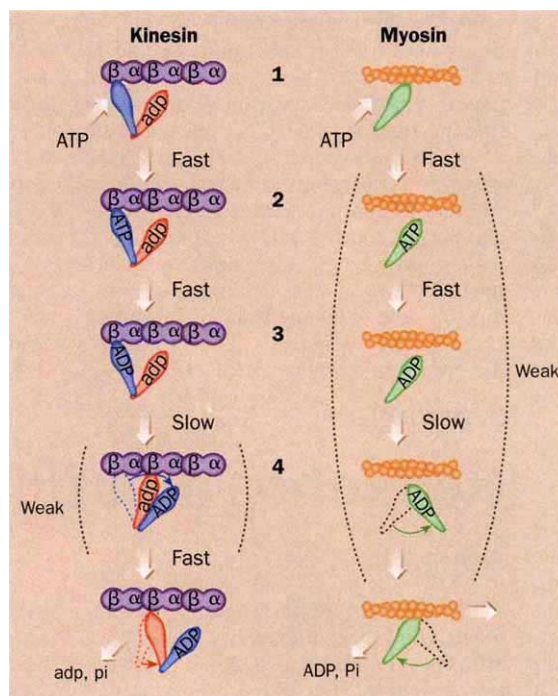
separate the vesicle from the microtubule if there were, for kinesin, a prolonged weakly bound state in the mechanochemical cycle similar to that of the myosin cycle. But some weakly bound state must occur: any force generator has to dissoci-

diffusing away, or even switching to a neighbouring protofilament just four nanometres away? This is the question addressed by the new studies, which show that for molecular motors, like people, it can pay to put heads together.

Both kinesin and myosin in the native state are two-headed molecules. Each has a pair of heavy chains bound together by an α -helical coiled-coil interaction. Each heavy chain has a globular 'head' domain (340 amino acids for kinesin¹²) which is both necessary and sufficient for converting ATP hydrolysis into movement. Each mechanochemical head contains one ATP-binding site and as-yet undefined sites which interact with the cytoskeletal filament. For myosin, it is likely that the heads normally act independently⁷, although this is still unproven. The new work by Berliner *et al.*² and by Gilbert *et al.*¹ now proves what has been suspected for some time^{3,4,13}: for kinesin, the two heads of the dimer interact, and this cooperativity is what enables a single native motor to track for long distances along a microtubule protofilament.

Berliner *et al.*² ask whether beads carrying a single-headed kinesin construct (a deletion mutant of kinesin missing the α -helical dimerization domain) are able to track along microtubule protofilaments like recombinant dimeric molecules having two globular heads. Using video-enhanced light microscopy to record the movement of beads carrying recombinant kinesin molecules as they translocate along microtubules *in vitro*, they follow the side-to-side motion of the beads using a nm-scale tracking technique applied to digitized images¹⁰. Their results show that beads carrying even one single recombinant two-headed kinesin molecule (K448 or K401) can track along one protofilament for long distances, similar to native kinesin. In contrast, beads carrying a single-headed kinesin construct (K340) appear to wander over the entire surface of the microtubule as they move axially. The prerequisite dimer of kinesin heads probably walks 'arm-over-arm', with one arm always attached (see figure). It is interest-

ing that although a single dimeric motor can track in this way, no bead movement could be detected at dilutions of the single-headed construct at which only one head is bound per bead. This negative result suggests that each kinesin head operates like a myosin in having a



Comparison of kinesin and myosin⁷ mechanisms. The figure shows, for each motor, a single mechanochemical cycle, where one ATP is hydrolysed and one 'step' along the cytoskeletal filament occurs. Unlike myosin, which is weakly bound (dotted brackets) to actin during most (~95%) of the cycle⁷, kinesin is tightly bound to the microtubule during most of the cycle and therefore a single motor moves processively for long distances. The mechanisms differ in that myosin heads are presumed to act independently, so only one head is represented in the figure, whereas for kinesin, the two equivalent heads (blue and red) cooperate^{1,2,17} and walk hand-over-hand along a single microtubule protofilament. Unlike myosin, where ATP binding (step 1) leads to rapid dissociation of myosin from actin (step 2) before hydrolysis (step 3), Gilbert *et al.*¹ show that for kinesin, the weakly bound state occurs after hydrolysis and is very brief. In the figure, ATP hydrolysis and microtubule binding alternate between the two kinesin heads, although this idea is still unproven¹⁷. Nucleotides bound by each of the two kinesin heads are represented as upper or lower case to illustrate alternating catalysis, whereby a single 8-nm step is associated with ATP binding and hydrolysis in one head (upper case) and product release from the other head (lower case). The major conformational changes (step 4) leading to movement are hypothetical, although it is likely that these occur during this slow kinetic step¹.

ate from the cytoskeletal polymer after the power stroke so that the initial conformation can be reset without reversing the work done on the filament during the power stroke. So how can a single molecule like kinesin step several hundred times in succession without letting go and

prolonged 'off' time to allow for resetting the pre-powerstroke conformation. An obvious task is to measure displacements and forces from one single kinesin head in order to prove this idea.

The results of Gilbert *et al.*¹ provide independent evidence that the two heads of a kinesin dimer cooperate, and that this cooperativity keeps kinesin attached to the microtubule. Using stopped-flow kinetic procedures, they were able to measure the key rate constants for the interaction of a dimeric kinesin construct (K401) with ATP and microtubules, including the rate of kinesin release from microtubules. Their main finding is that the release of K401 from microtubules has a rate of 13 s^{-1} , whereas the steady-state turnover rate of ATP hydrolysis is 20 s^{-1} . To reconcile how one step in the cycle, namely release from the microtubule, can be slower than the entire ATP hydrolytic cycle, the authors propose that the two heads must release from the microtubule sequentially, not randomly. Computer simulation shows that two heads, each hydrolysing ATP at a rate of 20 s^{-1} , would lead to an overall release rate of both heads of 13 s^{-1} , in agreement with the measured rate.

The elegant study of Gilbert *et al.* contributes additional information about how kinesin works. Most important, their analysis proves what has been suspected for some time^{14,15}: that the mechanochemical cycle for kinesin is fundamentally different from that of myosin or dynein, adapting to kinesin's special role as a soluble motor, in contrast to myosin or dynein, which operate in a structural complex (the sarcomere or axonemes, respectively). The most distinctive feature of kinesin mechanochemistry is that ATP binding and hydrolysis within the active site occurs long before the motor dissociates from the microtubule. This is in marked contrast to myosin, in which bind-

ing of ATP to myosin rapidly releases the motor from the actin filament, and where hydrolysis occurs while the motor is dissociated or weakly bound¹⁶.

Although the Gilbert *et al.* findings leave little doubt that the two kinesin heads interact, their proposal that the heads release sequentially, while accounting for the biochemical measurements, does create difficulties in explaining movements, because it is difficult to imagine how the first head could generate a power stroke leading to an 8-nm step⁵ if the second head is still tightly bound to the microtubule. Here, another recent paper¹⁷ may offer a solution. Based on the finding that a dimeric (K392) kinesin construct retains one tightly bound ADP when it is attached to a microtubule, whereas a monomeric kinesin (K340) releases all bound ADP when combined with microtubules, Hackney¹⁷ proposes that the two heads catalyse ATP hydrolysis and movement in an alternate fashion (see figure). Unfortunately, the stoichiometry of ADP release as

measured by Hackney disagrees with the phosphate burst results of Gilbert *et al.*, who find that the amount of phosphate formed during the initial hydrolysis by kinesin dimer is one mole of inorganic phosphate (P_i) per mole of heads, not 0.5 mole of P_i for each mole of kinesin heavy chain as would be expected from Hackney's studies.

In summary, although the evidence is now strong that kinesin heads cooperate and it is likely that this involves an 'arm-over-arm' activity, the exact mechanism is still controversial. In the near future, we can expect that the solution of the atomic structure of the kinesin motor domain, together with further biophysical and rate measurements applied to two-headed and single-headed kinesin constructs, will lead to a definitive model for how kinesin works. □

Bruce J. Schnapp is in the Department of Cell Biology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115, USA.

DEVELOPMENTAL BIOLOGY

Hedgehog digs up an old friend

Seth S. Blair

FROM the start of the century, embryologists have been uncovering evidence of diffusible morphogens, signalling molecules used by cells to pattern the development of adjacent tissues. Of those now known, few have generated as much excitement as the recently discovered *Drosophila* protein Hedgehog (Hh) and its various vertebrate homologues (reviewed in refs 1–3). Three studies on pages 705, 709 and 711 of this issue^{4–6}, and three more in this week's issue of *Cell*^{7–9}, now combine to provide important new information about how Hh works. The most surprising of these results is that reception of the Hh signal appears to depend upon an old friend: protein kinase A (PKA)^{4,6–9}, the principal intracellular sensor for the second messenger cyclic AMP (reviewed in refs 10, 11).

The molecular nature of morphogens and their mode of action has remained one of the deepest mysteries of developmental biology, and it is only recently that a number of good candidates for such molecules have been discovered. Much of the fascination with Hh-like proteins arises from the variety of patterning events they appear to control. In *Drosophila*, Hh was originally identified for its role in maintaining the polarity of embryonic segments^{1–3}; its expression by posterior cells in each segment is required for proper gene expression and patterning in cells just to the anterior. Additional Hh-dependent events have since been

revealed, and two of the new studies provide further evidence for Hh's involvement in the development of the *Drosophila* eye^{4,5}. In vertebrates, the list of tissues that express Hh-like proteins reads like a *Who's Who* of famous inducers (notochord, floor plate, zone of polarizing activity, to name a few), and the proteins alone can often mimic the effects of these tissues¹.

How are these dramatic effects brought about? The manner in which the Hh signal is received is as yet only poorly understood. Although it does have proteolytic properties¹², Hh does not fall into any previously known class of signalling molecules. And there is no proven Hh receptor — the *Drosophila* transmembrane protein Patched (Ptc) is the only plausible candidate so far^{1,2,13}. Loss of Ptc results in a phenotype resembling one due to ectopic misexpression of Hh, but this phenotype is only slightly disrupted by the loss of Hh. Thus, Hh acts by inactivating or counteracting some aspect of Ptc function, either indirectly or by binding to Ptc.

The new studies show that the loss of the PKA catalytic subunit in *Drosophila* also results in a phenotype which strongly resembles, but is independent of, reception of the Hh signal^{4,6–9}. Reduction of PKA activity can also partially rescue weak Hh phenotypes, indicative of a quantitative interaction^{4,7,9}. Thus, Hh appears to act by counteracting a PKA-dependent process (see figure).

1. Gilbert, S. P., Webb, M. R., Brune, M. & Johnson, K. A. *Nature* **373**, 671–676 (1995).
2. Berlinger, E., Young, E. C., Anderson, K., Mahtani, H. K. & Gelles, J. *Nature* **373**, 718–721 (1995).
3. Howard, J., Hudspeth, A. J. & Vale, R. D. *Nature* **342**, 154–158 (1989).
4. Block, S. M., Goldstein, L. S. B. & Schnapp, B. J. *Nature* **348**, 348–352 (1990).
5. Svoboda, K., Schmidt, C. F., Schnapp, B. J. & Block, S. M. *Nature* **365**, 721–727 (1993).
6. Finer, J. T., Simmons, R. M. & Spudis, J. A. *Nature* **368**, 113–119 (1994).
7. Spudis, J. A. *Nature* **372**, 515–518 (1994).
8. Uyeda, T. Q. P., Warrick, H. M., Kron, S. J. & Spudis, J. A. *Nature* **352**, 307–311 (1990).
9. Uyeda, T. Q. P., Kron, S. J. & Spudis, J. A. *J. molec. Biol.* **214**, 699–710 (1990).
10. Gelles, J., Schnapp, B. J. & Sheetz, M. P. *Nature* **331**, 405–408 (1988).
11. Ray, S., Meyhofer, E., Milligan, R. A. & Howard, J. J. *Cell Biol.* **121**, 1083–1093 (1993).
12. Stewart, R. J., Thaler, J. P. & Goldstein, L. S. B. *Proc. natn. Acad. Sci. U.S.A.* **90**, 5209–5213 (1993).
13. Schnapp, B. J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **87**, 10053–10057 (1990).
14. Lasek, R. J. & Brady, S. T. *Nature* **316**, 645–647 (1985).
15. Romberg, L. & Vale, R. D. *Nature* **361**, 168–170 (1993).
16. Lynn, R. W. & Taylor, E. W. *Biochemistry* **10**, 4617–4624 (1971).
17. Hackney, D. D. *Proc. natn. Acad. Sci. U.S.A.* **91**, 6865–6869 (1994).

These unexpected results were obtained by examining the role of Hh in two post-embryonic events: the patterning of the appendages and of the compound eye. The leg, wing and eye of *Drosophila* develop from imaginal discs, epithelial sacs which are set aside during embryonic development and grow throughout larval life, forming the patterned ectodermal tissues of the adult after metamorphosis^{1,3}. During much of the early period of leg and wing disc development, Hh is expressed in the posterior 'lineage compartment' of each disc. Another set of proteins critical to wing

express Hh, which diffuses anteriorly and induces expression of Dpp in more anterior cells. Unlike the situation in the wing and leg, Dpp in turn triggers differentiation, another round of Hh expression, and so on^{18,19} (see figure). Ectopic, anterior Hh expression is sufficient to induce precocious Dpp expression and differentiation, and these cells can act in turn as organizing centres driving progressively more distant differentiation^{4,5}. Once again, the localized removal of PKA mimics these effects^{4,7}.

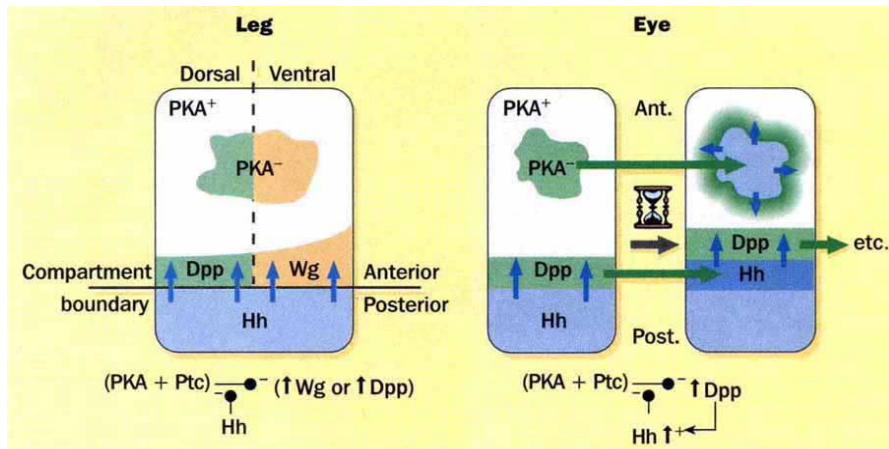
How is the signalling pathway ordered? Without more genetic and, especially,

Ptc: Ptc could reinforce and Hh counteract some process requiring only basal PKA activity. The available evidence for the leg and wing supports the latter suggestion. If Hh and Ptc act solely by regulating cAMP, then the PKA activity needed to rescue PKA⁻ cells should also rescue Ptc⁻ cells and disrupt normal Hh signalling. In fact, expression of a constitutively activated, cAMP-independent PKA can disrupt Hh signals and rescue Ptc⁻ cells, but only at levels far in excess of those needed to rescue PKA⁻ cells^{8,9}.

These results are more consistent with competition between Hh and PKA, as would be found if PKA were phosphorylating while Hh was stimulating dephosphorylation of a common target, for example⁸. Whatever the true picture, however, PKA must have a substrate: obvious possibilities include the Fused serine/threonine kinase and the putative transcription factor Ci-D. Both are required for Hh signalling in the embryo², and Ci-D contains potential PKA phosphorylation sites²⁰.

How do morphogens pattern tissues as large as a wing or leg? What is the range of such molecules? It should be noted that anterior leg and wing cells lacking PKA undergo not only cell-autonomous changes in cell identity, but also induce the dramatic repatterning of quite distant wild-type cells, including duplications and ectopic distal outgrowths^{6,9} similar to those induced by ectopic Hh expression^{12,21}. Hh is not ectopically expressed in PKA⁻ cells; double-mutant experiments indicate that these effects are almost entirely caused by the ectopic expression of two other secreted morphogens, Dpp and Wg⁷⁻⁹. Nor does it end here, as in the leg the combination of dorsal Dpp and ventral Wg at the distal tip apparently evokes other factors responsible for the proximo-distal axis^{7-9,21,22}. Thus, instead of acting itself over a long range, one morphogen may trigger the adjacent expression of another. It will be interesting to see how much further this kind of 'bucket-brigade' patterning can extend. □

Seth S. Blair is in the Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706, USA.



Models of Hedgehog action in *Drosophila* leg and eye. *Top*, the induction of anterior Wingless or Decapentaplegic expression by posteriorly secreted Hedgehog, and the similar response in cells lacking protein kinase A. *Bottom*, the formal genetic interactions; both PKA and Patched are required to repress Dpp or Wg expression. In the leg the response to Hh signal or to the removal of PKA and/or Ptc is limited to the anterior compartment, and differs in dorsal and ventral domains; in the wing (not shown) the situation resembles that in the dorsal leg. In the eye the anterior expression of Dpp in turn induces anterior differentiation and Hh expression, resulting in the posterior to anterior progression of differentiation.

and leg patterning, including the growth-factor-like molecules Wingless (Wg, of the Wnt family) and Decapentaplegic (Dpp, of the TGF- β family), are expressed just to the anterior of the compartment boundary. As in the embryonic segment, this patterned anterior gene expression is controlled by Hh diffusing from the posterior; only cells of the anterior compartment can respond to this signal (reviewed in refs 14, 15; see figure). Ectopic Hh expression¹⁴⁻¹⁶ or loss of Ptc^{6,8,9,17} in anterior cells results in ectopic Dpp and Wg expression and the ectopic formation of tissue types normally found just to the anterior of the compartment boundary. Surprisingly, removal of PKA from anterior cells mimics these effects, even in the absence of Hh function⁹ and without inducing ectopic Hh expression⁶⁻⁹.

Nor is this finding peculiar to interactions between compartments. The developing eye disc of *Drosophila* lacks anterior and posterior compartments; instead, the differentiation of the individual optic elements of the compound eye moves progressively from posterior to anterior. This progression occurs in part because the more fully differentiated cells

biochemical evidence, many types of linear and parallel pathways are possible. Even putting the cytoplasmic PKA downstream of the transmembrane Ptc is perilous. In other systems, PKA activity is controlled by cAMP, which binds to the enzyme's regulatory subunit and frees the catalytic subunit to phosphorylate a variety of substrates, thereby modulating cellular responses such as transcription or cell division^{10,11}. Thus, in the most tempting scenario, Hh and/or Ptc would directly regulate PKA activity by controlling the levels of cAMP. Alternatively, PKA activity might not depend on either Hh or

- Fietz, M. J. *et al.* *Development Suppl.* 43–51 (1994).
- Forbes, A. J., Nakano, Y., Taylor, A. M. & Ingham, P. W. *Development Suppl.* 115–124 (1993).
- Lawrence, P. A. & Morata, G. *Cell* **78**, 181–189 (1994).
- Strutt, D. I., Wiersdorff, V. & Mlodzik, M. *Nature* **373**, 705–709 (1995).
- Heberlein, U., Singh, C. M., Luk, A. Y. & Donohoe, T. J. *Nature* **373**, 709–711 (1995).
- Lepage, T., Cohen, S. M., Diaz-Benjumea, F. J. & Parkhurst, S. M. *Nature* **373**, 711–715 (1995).
- Pan, D. & Rubin, G. M. *Cell* **80**, 543–552 (1995).
- Jiang, J. & Struhl, G. *Cell* **80**, 563–572 (1995).
- Li, W., Ohlmeier, J. T., Lane, M. E. & Kalderon, D. *Cell* **80**, 553–562 (1995).
- Ray, S. S., Buechler, J. A. & Yonemoto, W. A. *Rev. Biochem.* **59**, 971–1005 (1990).
- Hartwell, L. *Nature* **371**, 286 (1994).
- Lee, J. J. *et al.* *Science* **266**, 1528–1537 (1994).
- Bejsovec, A. & Weischaus, E. *Development* **119**, 501–517 (1993).
- Basler, K. & Struhl, G. *Nature* **368**, 208–214 (1994).
- Tabata, T. & Kornberg, T. B. *Cell* **76**, 89–102 (1994).
- Felsenfeld, A. L. & Kennison, J. A. *Development* **121**, 1–10 (1995).
- Capdevila, J., Estrada, M. P., Sanchez-Herrero, E. & Guerrero, I. *EMBO J.* **13**, 71–82 (1994).
- Heberlein, U., Wolff, T. & Rubin, G. M. *Cell* **75**, 913–926 (1993).
- Ma, C., Zhou, Y., Beachy, P. A. & Moses, K. *Cell* **75**, 927–936 (1993).
- Orenic, T. V., Slusarski, D. C., Kroll, K. L. & Holmgren, R. A. *Genes Dev.* **4**, 1053–1067 (1990).
- Diaz-Benjumea, F. J., Cohen, B. & Cohen, S. M. *Nature* **372**, 175–179 (1994).
- Campbell, G., Weaver, T. & Tomlinson, A. *Cell* **74**, 1113–1123 (1993).

Fast-focus telephoto eye

Michael F. Land

THOSE of us who still own a reflex camera that allows us to do our own focusing will know that it can also be used as a convenient measure of distance: after bringing an object into focus, we can read off the setting on the focus ring. A new technique for measuring accommodation in living eyes, described on page 692 of this issue¹, reveals that the chameleon uses an analogous method to determine distance. The state of focus of the eye lens acts as its principal distance cue, and not only is the focusing mechanism of the chameleon lens very fast and its range unusually extensive, it is also accurate enough to supply the muscles of the projectile tongue with the information they need to fire the tip just the right distance to zap the insect prey.

What makes chameleons different from most other predatory vertebrates is that they use this mechanism to the exclusion of others. Binocular animals like ourselves have powerful strategies based on triangulation: the amount the eyes have to converge on a target supplies some information, and disparity — the extent of the mismatch of the images in the two eyes — supplies most of the rest. As a last resort, we can still get a little information from focus (as in threading a needle with one eye closed without moving your

head), but it is at best approximate. Although in the last moment before they strike, chameleons do fixate with both eyes, they still do not triangulate: a pair of prisms in front of the eyes does not affect their judgement, but a lens that shifts the plane of focus does² (Fig. 1).

Chameleon eyes are peculiar in other respects. They are famous for moving independently, at least until they lock onto prey. They also look like rotating turrets, because the eyelids make an aperture in front of the cornea which serves as an iris and moves with the eye. The real significance of the unusual positioning of the iris may become clear when we consider the other remarkable conclusion from Ott and Schaeffel's paper¹, which is that the lens of the chameleon eye, although slightly biconvex, has negative power when unaccommodated. This is astonishing, and as far as I know unique among animals. The simple physical explanation would be that the lens is of lower refractive index than the humours of the eye, but this cannot be right because there are no plausible cellular constituents that could achieve this. Alternatively, and undoubtedly correctly, the outer lens must disguise a concave lens within it.

What is disturbing here is that nearly all vertebrate lenses, from those of fish to our own, contain refractive index gradients that provide internal lenses with more positive power than the external biconvex shape suggests. Why should chameleons be different, and how do they achieve this strange optical feat? One possible way would be to reverse the usual vertebrate pattern of a lens with a high index in the centre, and instead put the high-index material into the periphery. The lenses are flanked by structures called lens pads, which are unusually large in chameleons compared with other reptiles. If these had a higher refractive index and were deformable, they might have a role both in the negative unaccommodated power of the lens as well in accommodation itself. But as yet there is little to go on, and this remarkable lens beckons further study.

Why have a negative lens at all? Ott and Schaeffel make two suggestions. First, the negative starting point gives the lens a greater accommodative range, which chameleons undoubtedly use. Second, by having a negative lens behind a positive lens (the cornea), the optical system assumes a classic telephoto design, a combination that has a focal length longer than its physical length (Fig. 2a). This makes the image magnification larger for a given

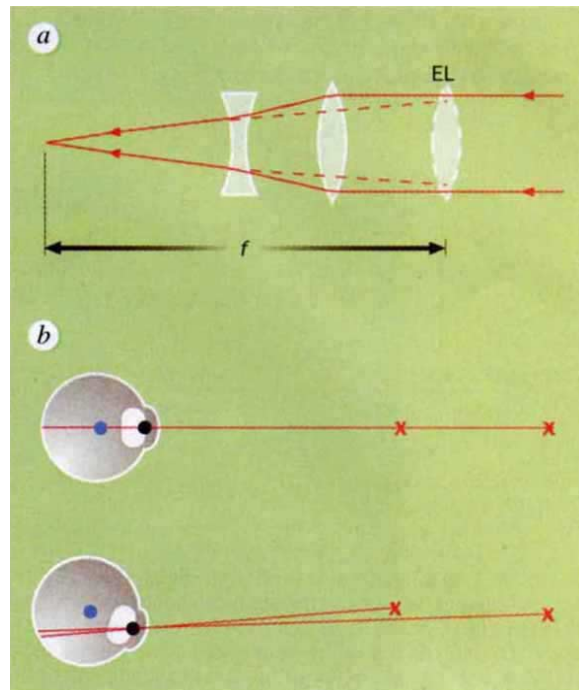


FIG. 2 a, The telephoto principle. The presence of a negative lens increases the effective focal length (f), and places the apparent position of an equivalent lens (EL) in front of the actual lenses. b, Diagram showing that rotation of an eye in which the nodal point (dot) does not coincide with the centre of rotation (circle) causes the images of objects at different distances to move relative to each other. For a 10-mm eye with the geometry shown, a 20° rotation will cause objects at 1 and 2 m to separate by 8.6 μ m, which corresponds to several cone diameters.



FIG. 1 Photograph by Lindesay Harkness of a chameleon (*Chameleo jacksoni*) wearing spectacles. Lenses that shift the plane of focus cause incorrect distance judgements, whereas prisms do not. (Reproduced from ref. 2, with permission.)

eye size, and for an animal that makes use of fine detail in prey capture, this is obviously valuable. Something rather similar also occurs in the eyes of hawks³, but here the negative element is in the retinal surface, not the lens.

I think there may be a third reason for the negative lens, which also follows from the telephoto idea. This is that the negative lens pushes the nodal point of the eye a long way forward, close to the cornea, and incidentally close to the location of the chameleon's weird eyelid iris. (The nodal point is the point in the eye through which straight lines can be drawn that map objects onto images; it is where a single

1. Ott, M. & Schaeffel, F. *Nature* **373**, 692–694 (1995).
2. Collett, T. S. & Harkness, L. I. K. in *Analysis of Visual Behavior* (eds Ingle, D. J., Goodale, M. A. & Mansfield, R. J. W.) 111–176 (MIT Press, Cambridge, MA, 1982).
3. Snyder, A. W. & Miller, W. H. *Nature* **275**, 127–129 (1978).

thin lens could be placed to mimic the whole optical system.) In our eyes, the nodal point is close to the eye's centre of rotation, and this means that when we rotate our eyes all objects in a straight line stay in a straight line, whatever their distance. But if the nodal point is a long way in front of the centre of rotation, two objects that start off in a straight line become separated when the chameleon rotates its eye (Fig. 2b). For an animal that

stays camouflaged and still, and hunts monocularly, the small image displacements that eye movements produce may allow foliage at different distances to be distinguished and, along with the leaves, the creatures that live on them. □

Michael F. Land is at the Sussex Centre for Neuroscience, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK.

MOLECULAR EVOLUTION

Life at the margins

James P. Ferris

WHAT led to life on Earth? So great a question can only be tackled by breaking it down into sections. On page 683 of this issue a group led by Stanley Miller¹ does just that, by looking at the possible prebiotic synthesis of the coenzyme pantetheine. Their experiments show that this biologically active molecule could plausibly have formed in the sunbaked sludge left at the margins of drying pools and lagoons.

In modern life-forms, coenzymes are small molecules which in association with enzymes catalyse redox and group transfer reactions. Their structures vary widely, in marked contrast to those of the repetitive structural units present in proteins and nucleic acids. Many contain nucleotides as structural units including nicotinamide adenine dinucleotide (NAD⁺) or flavin adenine dinucleotide (FAD) but some, such as biotin or lipoic acid, bear no resemblance to nucleic acids.

There are at least two views of the prebiotic synthesis of coenzymes. One is that at an early stage of evolution they were part of the covalent structure of RNA, rather than standing alone. They assisted in an RNA-based metabolism (now called the RNA world) before the evolution of protein catalysis². With the advent of the protein-DNA world, proteins which could bind coenzymes were produced so that the metabolic processes they catalysed could continue.

A second view is that the structural units present in coenzymes were formed independently, in chemical processes and yields comparable to those that resulted in the synthesis of nucleotides and amino acids. This hypothesis is supported by the observation that many coenzymes contain nucleotide units or appear to have been derived from nucleotides. From this it is concluded that coenzymes formed because their precursors were abundant on the primitive Earth. In this view, ribozymes (RNA enzymes) evolved which could bind these useful structures and use them in metabolic processes in

the RNA world. This view is supported by the reports that RNAs have been produced by directed evolution in the laboratory which bind NAD⁺, FAD, nicotinamide mononucleotide and adenosine triphosphate^{3,4}, although these complexes don't, at present, show catalytic activity.

Here, Keefe *et al.*¹ base their 'prebiotic' synthesis of pantetheine on the second view, the presumed abundance of its component molecules, pantoic acid, β -alanine and cysteamine on the primitive Earth. Modest heating (40–118 °C) of a dried mixture of these three substances gives pantetheine in up to 1 per cent yield, in a day to more than six weeks depending on temperature. The authors note that all three pantetheine precursors are very water soluble, and they propose that these species would be among the last to precipitate when a pond or lagoon dried out and the residue of organics remaining was further heated. Consequently, the molecules would be in close proximity so that these solid-state reactions would occur. The authors do not report the products of the reaction mixture in which pantoic acid was used in place of pantoic lactone, but one would expect that cyclization of the hydroxyacid to the lactone would occur⁵ under the dry heating conditions used, and the lactone would initiate the synthesis of pantetheine.

Keefe *et al.* then attempted to convert pantetheine to the nucleotide derivative dephosphocoenzyme A by heating it with ATP or ADP. But this proved unsuccessful. Phosphodiester bonds are not formed readily from di- or triphosphates under prebiotic conditions, a result which suggests either that phosphodiester groups were rare on the primitive Earth or that they formed using phosphate activating groups other than the pyrophosphate group.

This research is one of a very few papers dealing with the prebiotic synthesis of coenzymes. Proposed prebiotic syntheses of nicotinamide and its derivatives were reported^{6,7} in the early 1970s and the formation of NAD⁺ from the correspond-

ing nucleotides was reported⁸ in 1978, but the syntheses bear little relation to the current one by Miller's group.

Of course, the synthesis of coenzymes from their components is only part of the story. How did the precursors themselves arise on the early Earth? Miller and co-workers have given this matter their attention on previous occasions, and have suggested prebiotic syntheses for cysteamine and pantoic acid, the starting materials for the formation of pantetheine and pantoic acid^{9,10}. Cysteamine is formed by the reaction of ethylene sulphide with ammonia. The prebiotic synthesis of ethylene sulphide has not been demonstrated but it is proposed to have been formed by the reaction of sulphur atoms with ethylene. The precursor to pantoic acid, pantoic acid, is formed by the reaction of formaldehyde and HCN with isobutyraldehyde. It is assumed that isobutyraldehyde is formed by passing an electric discharge through methane, nitrogen, ammonia and water, as the corresponding amino acid, valine, is observed as a product.

It is surprising that there have been so few reports of the prebiotic syntheses of coenzymes, as they are often assumed to have been present on the primitive Earth. Perhaps, contrary to the evidence presented in this report, most coenzymes were not synthesized readily from their component parts on the primitive Earth but are the products of an early form of life. Another possible explanation is that as most coenzymes contain a nucleotide link, more data concerning the prebiotic synthesis of nucleotides is required before we can begin to understand the prebiotic formation of coenzymes. Or perhaps the reason is simply that, as most scientists in the field of the origins of life are currently investing their time in the synthesis of oligonucleotides and oligopeptides, few have seriously addressed the question of the prebiotic formation of coenzymes. I would suspect the latter — but surely these exotic molecules deserve more attention. □

James P. Ferris is in the Department of Chemistry, Rensselaer Polytechnic Institute, Troy, New York 12180, USA.

1. Keefe, A. D., Newton, G. L. & Miller, S. L. *Nature* **373**, 683–685 (1995).
2. White, H. B. *J. molec. Evol.* **7**, 101–104 (1976).
3. Burgstaller, P. & Famulok, M. *Angew. Chem. Int. Edn Engl.* **33**, 1084–1086 (1994).
4. Saassanfar, M. & Szostok, J. W. *Nature* **264**, 550–552 (1993).
5. Fridman, Y. D. *et al. Russ. J. inorg. Chem.* **30**, 16–19 (1985).
6. Dowler, M. J. *et al. Science* **169**, 1320–1321 (1970).
7. Friedmann, N. *et al. Science* **171**, 1026–1027 (1971).
8. Lohmann, R. & Orgel, L. E. *J. molec. Evol.* **11**, 17–23 (1978).
9. Miller, S. L. & Schlesinger, G. *J. molec. Evol.* **36**, 302–307 (1993).
10. Miller, S. L. & Schlesinger, G. *J. molec. Evol.* **36**, 308–314 (1993).

Exploding volcanic myths

Jonathan Fink

FOR students of explosive volcanism, the 1990s have been a time of unparalleled accomplishment tempered by tragic loss. New modelling experiments from two groups of workers may now help us to come up with better ideas of when volcanoes are due to erupt violently, lessening their deadly impact. Mader *et al.*¹ published their results late last year; those of Sugioka and Bursik² appear on page 689 of this issue.

Geologists have had two notable successes in their recent attempts to mitigate the hazards of erupting volcanoes. The most dramatic was in 1991 at Mount Pinatubo in the Philippines, where timely warnings saved thousands of lives and

billions of dollars' worth of military equipment. Last September's reawakening of Rabaul Caldera in Papua New Guinea prompted an evacuation that greatly reduced casualties. In both cases, scientists were able to notice familiar patterns of deformation and seismicity and then explain the significance of this activity to responsible civil officials. This recognition came from studying what had occurred at eruptions elsewhere, and also from predictions of what might happen locally based on theoretical models.

Over this same period, smaller explosive eruptions have been more difficult to anticipate, and both scientists and civilians have paid the price. Comparatively

mild outbursts from Galeras in Colombia, Unzen in Japan and Guagua Pichincha in Ecuador have taken the lives of eleven volcanologists. Re-examination of seismic and gas data from the days before the Galeras eruption revealed correlations that are now being used to look for future explosions of this type³. The Unzen lava dome, whose collapse gave rise to deadly pyroclastic surges, had been carefully monitored for months. At Pichincha the two scientists killed had received a warning based on seismicity an hour before. Thus the deaths arose not so much from lack of information as from the inability to interpret it correctly. An important lesson that has been learned from these fatalities is that better theoretical models are needed to tell us which data to collect and what such numbers mean.

Most geologists agree that the triggering of explosive eruptions involves certain

OBITUARY

Adolf Butenandt (1903–1995)

ADOLF Butenandt, the outstanding biochemist in Germany in the first half of this century, died in Munich on January 18. Given the methods of the time, his achievements in isolating hormones were truly towering.

Butenandt studied chemistry and biology in Marburg and Göttingen, and did his PhD thesis under the supervision of Adolf Windaus. As a young PhD, he was appointed at the Institute of Organic Chemistry in Göttingen, under Windaus, to start work on the female sex hormone, now known as oestrone. He isolated this hormone in the summer of 1929, simultaneously with but independent of Edward Doisy, and by 1932 had already worked out its chemical structure. He then turned to the male sex hormone androsterone, which he isolated from men's urine. Time-consuming purification procedures, guided by a bioassay, were necessary to obtain crystals of the hormone. The structure followed shortly afterwards: androsterone also turned out to be a steroid.

The next task was progesterone; this time the starting material was pig's ovaries. Again, difficult purification procedures monitored by a bioassay were necessary. In the final step, a chemical reaction (formation of a semicarbazone) helped to achieve crystallization. The results were published in 1934. So, within a mere five years Butenandt had isolated three important hormones. For this work he received the Nobel Prize for Chemistry in 1939, but the Nazi government prevented him from accepting the honour.

Butenandt then left the field of steroid hormones and began to work on the genesis of insect eye pigments. Mu-

tants of *Drosophila* and the flour moth *Ephestia* were known that had light eyes because one component of the eye pigments was missing. Transplantation experiments had shown that a soluble substance was responsible for pigment



Butenandt (standing) at work with Ulrich Westphal on the isolation of progesterone in 1934.

formation. Butenandt followed up a hint that this substance might be a metabolite of tryptophan. He went on to discover that it was kynurenine, which led him to postulate that "genes act by providing an enzyme system that converts tryptophan to kynurenine". This was nothing other than the one-gene – one-enzyme hypothesis, published in 1940.

By this time, Butenandt had already risen to the position of director of the Kaiser Wilhelm Institute in Berlin-Dahlem. In 1944, the institute was transferred to Tübingen because of the war.

For his next project, Butenandt chose another problem in insect biochemistry: isolation of the hormone responsible for moulting. I was a participant in this research. The bioassay concerned was based on work by G. Fraenkel, and involved use of ligated larvae of the blowfly *Calliphora*. The starting material consisted of pupae of the silkworm, and in the summer of 1953 we bought up all silk cocoons available. We used cocoons of both sexes, the males for the moulting hormone, the females for the sex attractant. From 500 kg of pupae, we ended up with 25 mg of the crystalline hormone which we called ecdysone. The structure, when elucidated, came as a surprise. Ecdysone was another steroid hormone.

The silkworm's sex attractant is produced in small glands sitting at the tip of the female's abdomen, and is released to lure the male moths for copulation. Isolating it was again a fight for enough starting material. The great campaign of 1953 yielded 200,000 sex glands, but even that was not enough. Butenandt and his co-worker Hecker had to order 500,000 glands from Japan, and from this material the attractant was extracted and its structure determined. This was the first insect pheromone to be isolated.

In 1959, Adolf Butenandt was elected President of the Max-Planck-Gesellschaft der Wissenschaften (this was the new name for the former Kaiser-Wilhelm-Gesellschaft). He served in this position until his retirement in 1972.

Peter Karlson

Peter Karlson is a Professor Emeritus at the Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-35037 Marburg, Germany.

common stages: gases in the molten magma come out of solution to form bubbles, bubbles expand in response to a drop in pressure, the coherent melt breaks into fragments, and pieces of magma are expelled rapidly from the source. The sequence in which these events occur has not been firmly established, but one tenet has been widely accepted for over 15 years: once growing bubbles occupy roughly three-quarters of the volume of a magma body, it will blow itself apart⁴. Sophisticated numerical models that predict the likelihood and magnitude of future eruptions have been built around this assumption; petrologists reconstructing magma chamber conditions from explosive products have relied on it as well.

Suddenly, the validity of this maxim is being questioned widely. Field geologists looking at pumice under the microscope have described much larger bubble contents (up to 95 volume per cent) from lavas that failed to explode⁵, while theoreticians have shown that fragmentation should be possible at any bubble content greater than 50 per cent (D. M. Pyle, personal communication). Now results from the two sets of innovative laboratory simulations mentioned above^{1,2} are confirming the need for revised ideas of how explosive fragmentation works.

Modelling eruptive phenomena in the laboratory is an increasingly popular way to learn about dangerous processes that normally are hidden from view. Mader *et al.*¹ created a high-tech version of the most common analogy for the triggering of explosive eruptions — popping the top off a bottle of soda water. In one set of experiments, they abruptly released the pressure on a small confined tank of carbonated water and took high-speed motion pictures of the resulting evacuation. The standard eruption model predicts that after pressure release, a single wave of decompression should propagate downwards into the stagnant fluid, triggering bubbles to grow by diffusion and exsolution, followed by disruption of intervening melt and upward acceleration of the fragments. But the films show a different sequence. The drop in pressure causes bubbles to appear immediately throughout the solution, leading to rapid acceleration which makes the bubbles grow much more rapidly than would be possible by diffusion alone. In a second set of experiments, supersaturation was achieved by the physical mixing of K₂CO₃ and HCl solutions. In these flows, carbon dioxide exsolved heterogeneously, but again acceleration preceded bubble growth and fragmentation, rather than the reverse. In both cases, bubble concentrations at the time of disruption were highly variable.

In a complementary set of experiments described in this issue, Sugioka and Bursik² filled a volatile-charged bath with glass

spherules which could first act as bubble nucleation sites and then as simulated magma fragments. They observed two modes of expulsion depending on the initial vapour pressure of the condensed phase. At low volatile pressures, their results mimicked those of Mader *et al.*, with a finite period of pervasive nucleation followed by rapid bubble expansion that eventually drove out all of the material from the container. When the starting pressure exceeded a critical value, successive waves of vesiculation propagated downwards into the solution, separated from each other by discrete zones of compressed fluid in which bubble growth was suppressed. The resulting pulsatile ejection of particles, like that seen in many natural eruptions, had not been accounted for by previous theoretical or laboratory models.

These small-scale experiments have revealed potentially critical processes neither anticipated from theory nor incorporated into numerical calculations currently being used to predict the effects of future volcanic explosions. Additional insights might come from the opposite end of the experimental spectrum: intentional triggering of explosive eruptions in some remote area. One possibility would be to fire bullets, rockets or large quantities of

water at the oversteepened face of a heavily instrumented, growing lava dome, in order to record the sequence of events accompanying its explosive collapse⁶. Mount St Augustine, an isolated volcano in the Gulf of Alaska which has had dome eruptions in 1964, 1976 and 1986, would be a suitably remote place to plan such a campaign. The United States Department of Energy's National Laboratories, on the lookout for new socially relevant missions, could provide the expertise.

By incorporating the results of large- and small-scale experiments into their models, volcanologists may improve their ability to assess the dangers of future eruptions, both large and small. The outcome should be a reduction of the risks to populations surrounding volcanoes, and to themselves. □

Jonathan Fink is in the Department of Geology, Box 871404, Arizona State University, Tempe, Arizona 85287-1404, USA.

1. Mader, H. M. *et al.* *Nature* **372**, 85–88 (1994).
2. Sugioka, I. & Bursik, M. *Nature* **373**, 689–692 (1995).
3. Fischer, T. F. *et al.* *Nature* **368**, 135–137 (1994).
4. Sparks, R. S. J. *J. Volcanol. geotherm. Res.* **3**, 1–37 (1978).
5. Cashman, K. V. & Manghan, M. T. *Rev. Mineral.* **30**, 447–478 (1994).
6. Manley, C. R. *Workshop on Volcanic Disaster Prevention* (eds Newhall, C. G. & Tsumura, K.) 168 (Jap. Sci. Technol. Agency, Tsukuba, Japan, 1993).

TRANSCRIPTIONAL ACTIVATORS

Enter a polypeptide messenger

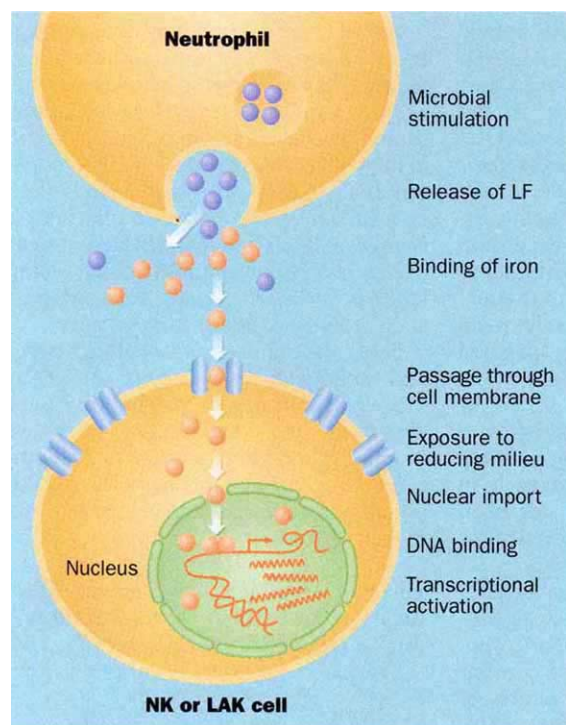
Patrick A. Baeuerle

THE profusion of pathways that convey different signals from outside the cell into the nucleus in order to alter the genetic programme all have one feature in common: their complexity¹. For instance, before the nuclear transcription factor Elk-1 is activated in response to a growth factor bound at the cell surface, a minimum of seven polypeptides have to interact sequentially to deliver the signal from the membrane receptor, through a succession of protein kinases, to the DNA-bound transcriptional activator². Fewer intermediaries are required to activate the cytoplasmic STAT transcription factors, which are phosphorylated directly by receptor-associated tyrosine kinases³. The simplest signalling system is that used by the large family of steroid hormone receptors in the nucleus: binding of a membrane-permeable ligand promotes their dimerization, DNA binding and transcriptional activity⁴. But an even neater courier service is now described by He and Furmanski on page 721 of this issue⁵, whereby an extracellular polypeptide acts as its own messenger, gaining entry into the cell nucleus to activate transcription directly.

That molecule is lactoferrin, a cationic

secretory protein with iron-chelating antimicrobial activity and specific DNA-binding properties. A cognate sequence, 5'-GGCACTTGC-3', is bound by dimers or tetramers of the protein with an affinity constant in the range of 1×10^8 M. When lactoferrin is added to the cell culture medium, it will transactivate a nuclear reporter gene harbouring one or four copies of the cognate sequence in the promoter; this effect is enhanced by iron-loaded lactoferrin. Two lines of evidence suggest that the action of lactoferrin is direct and not the work of a cell-surface-receptor-mediated pathway. First, lactoferrin is active in a cell-free transcription system; second, expression of a cytoplasmic form of lactoferrin devoid of its amino-terminal signal peptide is equally effective. Lactoferrin must therefore be considered as a secretory, but membrane-permeable transcription factor and DNA-binding protein.

An extracellular transcription factor has to overcome a number of hurdles to reach the DNA in the cell nucleus (see figure). Lactoferrin is stored in secretory granules of neutrophils until it is released by pathogen stimulation. Presumably, this stimulated release constitutes its major



Direct nuclear signalling by secreted lactoferrin (LF). The different stages of the pathway are indicated on the right. Blue particles, apo-LF; red particles, Fe-LF.

activating mechanism. Some of the lactoferrin is needed outside the cell to suppress microbial growth by chelating iron, but the rest makes its way across the lipid bilayer of the plasma or endosomal membrane of natural and lymphokine-activated killer cells (NK and LAK cells). Only a few such proteins have been described, including the cytoplasmic retroviral transactivators Tat (HIV)⁶ and Tax (HTLV-1)⁷. Tat and Tax can transactivate nuclear genes when added as recombinant proteins to cell culture medium. An interesting feature common to Tat, Tax and lactoferrin is that all three can chelate metal ions; in the case of Tat and Tax it is zinc. There is also some evidence that cell-surface receptors with their peptide ligands can make it directly to the nucleus to control gene expression⁸. How lactoferrin can be retained by the membranes of neutrophil granules but still traverse NK and LAK plasma membranes remains a paradox; perhaps only selected cells carry a membrane transporter protein for lactoferrin⁹, which would explain its cell-type-specific action.

Once lactoferrin has reached the cytosol, it faces a highly reducing environment which could rupture its 16 disulphide bonds. Chelation with iron may prevent any subsequent destabilization, which would explain why lactoferrin transactivates more actively in the iron-loaded form. The next hurdle is to get into the nucleus. He and Furmanski point out that there are stretches of basic amino acids in lactoferrin's amino terminus which may serve as nuclear location signal se-

quences⁵. Sequence-specific DNA binding comes next. Can lactoferrin efficiently occupy *cis*-acting DNA elements with an association constant of 1×10^8 M? A sufficiently high concentration of lactoferrin in the nucleus may compensate for this comparatively low affinity for DNA. Several questions remain: does lactoferrin have its own transactivation domain or must it recruit additional proteins, and which target genes does it control?

Signalling from the outside into the cell's nucleus usually involves binding of ligands to receptors, an optimal exploitation of regulatory potential, crosstalk between pathways, and compartmentation. Hence, I expect that secretory transcription factors that do not bother about cellular compartments and require very few additional proteins on their way to the nucleus,

remain exceptions to the rule. In the case of lactoferrin, perhaps, the exception provides a highly effective and rapid signalling mechanism between two cells in an emergency, namely the invasion of microbes. The evolutionary pressure to preserve a simple, archaic type of messenger may be very strong under such threatening conditions.

I wonder whether genetically altered variants of lactoferrin with a novel DNA-binding specificity and higher avidity can be designed. By simple intravenous injection, such artificial secretory transcription factors could, for instance, directly induce anti-oncogenes in malignant cells, or repress cytokine and cell-adhesion-molecule genes in blood or endothelial cells during systemic inflammatory syndromes. It is often the exceptional systems, which violate dogmas, that have the potential to advance science more than the ordinary ones. □

Patrick A. Baeuerle is in the Institute of Biochemistry, Albert-Ludwigs-Universität Freiburg, Hermann-Herderstrasse 7, D-79104 Freiburg, Germany.

1. Baeuerle, P. A. (ed.) *Inducible Gene Expression* Vols 1 and 2 (Birkhäuser, Boston, 1995).
2. Cahill, M. A., Janknecht, R. & Nordheim, A. in *Inducible Gene Expression* Vol. 2 (ed. Baeuerle, P. A.) 37–72 (Birkhäuser, Boston, 1995).
3. Fu, X.-Y. in *Inducible Gene Expression* Vol. 2 (ed. Baeuerle, P. A.) 99–130 (Birkhäuser, Boston, 1995).
4. Truss, M. & Beato, M. *Endocr. Rev.* **14**, 159–188 (1993).
5. He, J. & Furmanski, P. *Nature* **373**, 721–724 (1995).
6. Frankel, A. D. & Pabo, C. O. *Cell* **55**, 1189–1193 (1988).
7. Lindholm, P. F., Marriott, S. J., Gittlin, S. D., Bohan, C. A. & Brady, J. N. *New Biologist* **2**, 1034–1043 (1990).
8. Jans, D. A. *FASEB J.* **8**, 841–847 (1994).
9. Garre, C. et al. *J. Cell Physiol.* **153**, 477–482 (1992).

DAEDALUS

Gut feeling

MOTION sickness, says Daedalus, is a very odd phenomenon. Why should the body, subjected to mild swaying or rocking, decide to eject the contents of the stomach? Even more curious, the effect occurs only in vehicles. It is not triggered by the wildest and dizziest gymnastics. Daedalus reckons that the stomach is not merely a reservoir and food digester; it is a sense organ as well. Just as our ears detect vibrations from about 15 kHz down to 30 Hz, so the stomach handles the range between 30 and 0 Hz. The 'eardrum' for this sense is the diaphragm. Its upper surface can move quite freely within the chest, thanks to the compressible air in the lungs; its lower surface is joined to the stomach, which detects the movement. So the stomach 'hears' low vibrations and the inertial movements of the body. We sense the deepest throbbing notes of the organ mainly with the stomach, and we all know the eerie gastric lurch of inertial force in a suddenly dropping lift.

All this makes good evolutionary sense. To ease the problems of walking upright, the human stomach developed into a motion-sensing feedback system, telling the brain how well its orders have been obeyed. This neat arrangement came to grief when ships and vehicles were invented. In a pitching boat or swaying car, the stomach reports movements that the brain cannot reconcile with its own orders. It decides that the stomach is being perturbed by purely internal forces. These are usually caused by the bubbling of putrescent food, or distension from a blockage or undigested object. So the vomiting reflex is called in to remove the cause of the trouble. Hence the curse of motion sickness.

So, says Daedalus, motion sickness could be cured by arresting the gastric movements that cause it. One way would be to breathe, not air, but a solution of oxygen in an incompressible liquid. The lungs would lose their pneumatic elasticity, and would block the gastric surging that triggers motion sickness. Heroic volunteers have breathed oxygen solvents and lived to tell the tale, but most of us would prefer to be sick.

So DREADCO engineers are devising a friendlier system. The subject breathes air from a special pneumatic servo-unit which senses the displacements of his diaphragm, and raises or lowers the air pressure so as to cancel the movement. All the queasiness and discomfort of motion sickness will vanish instantly. The same principle should let the DREADCO pressure-compensated lift soar, sink and halt abruptly without terrifying the passengers.

David Jones

Anticipated stimuli across skin

SIR — Our perceptions occur only after the information we receive from our senses has been heavily preprocessed. The degree to which cognitive influences shape this processing has been debated for many years. Sensory saltation is a robust class of illusions that has been described as a shift in the perceived location of a stimulus towards a rapidly delivered subsequent stimulus^{1,2}. Our studies show that stimuli evoked in < 200-ms epochs are all mislocalized in sensory saltation. In the simplest two-stimulus form of the illusion, perceived stimulus locations are symmetrically shifted

which was properly localized) as a linear function of interstimulus interval (ISI), for ISIs below about 200 ms. For example, with an ISI of 100 ms, the second tap was perceived to be halfway between the actual loci of the two experimental taps, that is, mislocated by 5 cm.

A long-debated problem with Geldard's explanation of sensory saltation concerns how a brain mechanism can make a decision about where to locate a stimulus if the following 'attractor' has not yet occurred^{3,4}. Our findings demonstrate that this apparent paradox arose from a misunderstanding of the illusion.

Geldard and colleagues used trained subjects to reduce response variability. By training them, an important aspect of the illusion, a powerful biasing effect of directed attention (or expectation), was apparently circumvented.

We added a fourth stimulus at the second stimulus site as a second 'locator,' again well separated in time from the two experimental taps. In this simple symmetrical variation of Geldard's paradigm, naive subjects had no difficulty estimating the perceived separations between the two rapidly successive stimuli. However, although estimation of the absolute locations of the two stimuli on the forearm was quite variable, saltation stimuli were not perceived by naive subjects as one tap mislocalized towards the second, but rather, as taps located progressively closer to one another as the ISI was decreased (*a* in the figure). The centre points

between the two perceived taps were distributed as a roughly gaussian function centred at a point midway between the two actual stimulus locations (*b* in the figure).

A main source of the substantial variability in locating the two events on the skin was made apparent when naive subjects were asked to concentrate on the proximal (or distal) region of the forearm. When so instructed, perceived separations between the two taps did not change, but the perceived skin locations of the two taps were dramatically shifted up or down the forearm in the attended direction (*c* in the figure). The average difference between the centres of the perceived locations when naive subjects were instructed to attend to the proximal versus the distal forearm was 3.1 ± 0.47 cm (mean $\pm$ s.e., $P < 0.0001$).

With practice, this powerful effect progressively strengthened, and could result in stimulus mislocalization exactly as Geldard described — or in an equivalently great opposite-direction mislocalization.

Thus, although the perceived separations of stimuli were determined by their physical separations and ISIs, the perceived locations of the stimulus pairs were largely determined by a subject's selective attention and/or expectation. We conclude that, in training, Geldard's subjects were effectively taught to concentrate on the 'attractor' location to reduce response variability.

A better understanding of the cutaneous saltation illusion gives us further insight into the fundamentals of tactile and haptic perception. The mechanisms that generate cutaneous saltation (and presumably related phenomena in vision and audition) operate as a smoothing process by which the perceived distances between two events occurring within a short time domain are generated by symmetric convergence, with locations powerfully biased by ongoing cognitive estimates of where those events are most likely to have occurred.

Michael P. Kilgard

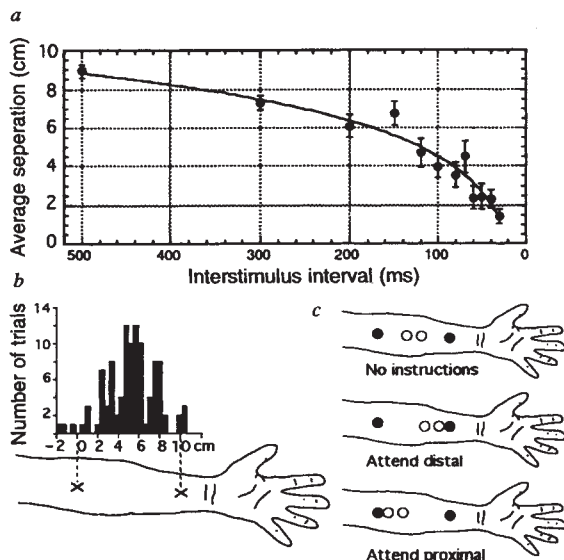
Michael M. Merzenich

Keck Center for Integrative Neuroscience,
University of California, San Francisco,
California 94143, USA

1. Geldard, F.A. & Sherrick, C.E. *Science* **178**, 178–179 (1972).
2. Geldard, F.A. *Sensory Saltation* (Erlbaum, New York, 1975).
3. Goodman, N. *Ways of Worldmaking* 74 (Hackett, Indianapolis, 1978).
4. Dennett, D. C. in *Consciousness Explained* 140–153 (Little, Brown, Boston, 1991).

Improved green fluorescence

SIR — The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has attracted widespread interest since the demonstration¹ that heterologous expression of the cloned gene² can generate striking green fluorescence. Despite the tremendous potential of recombinant GFP as a marker for gene expression or cell lineage or as an *in situ* tag for fusion proteins³, the wild-type protein from *A. victoria* has several significant deficiencies. Its excitation spectrum (*a* in the figure) shows peaks at both 396 and 475 nm. The longer-wavelength excitation peak has the advantages of greater photostability¹ and better matching to standard fluorescence filter sets, but is relatively low in amplitude. Considerable improvement should be possible, because a closely related protein⁴ from the sea pen *Renilla reniformis* has the same high quantum yield of emission (0.7–0.8), yet shows only one absorbance and excitation peak with an extinction coefficient per monomer more than 10 times that of the longer-



a, Perceived stimulus separation as a function of ISI. The forearm was hidden from the view of 13 naive subjects (Ss). After 10 repetitions of each ISI, Ss marked the locations of the two experimental taps with respect to the two locator taps. Bars are standard errors; $R = 0.97$. **b**, Distribution of centre points of experimental tap pairs, with data pooled for 30-, 40-, 50- and 60-ms ISIs. Mean location: 5.26 cm, s.d., 2.41 cm. **c**, Representative examples of the perception of the two locator and the two experimental stimuli for an ISI of 60 ms without (top) or with specific instructions that influenced their directed attention. Filled circles, actual stimulus locations; open circles, perceived stimulus locations.

ed towards one another. A misunderstanding of the fundamental nature of the illusion apparently arose because powerful effects of directed attention on absolute stimulus localization were not recognized.

Geldard and colleagues discovered sensory saltation and described versions of it in the tactile, auditory and visual systems studying its tactile form as their principal model². In their most basic tactile paradigm, a skin tap that served as a reference (the 'locator') was delivered 500 ms before the presentation of two experimental taps such that it did not influence the mislocalization. The first experimental tap was delivered at the locator site; the second 10 cm up or down the forearm. Geldard's subjects perceived the first experimental tap to be nearer to the second (the 'attractor',

wavelength peak of *Aequorea* GFP^{5,6}. We now report that simple point mutations in *Aequorea* GFP ameliorate its main problems and bring its spectra much closer to that of *Renilla*.

Serine 65 of the amino-acid sequence of *Aequorea* GFP becomes part of the ⁹p-hydroxybenzylidenemidazolone chromophore. To test the hypothesis⁷ that Ser 65 undergoes additional dehydration to form a vinyl side chain, we mutated that residue to Ala, Leu, Cys or Thr. If a vinyl group were formed by elimination of H₂O or H₂S, Ser and Cys should give identical spectra very different from Ala and Leu in which elimination is impossible. Serendipitously, all four mutants showed single excitation peaks, located at 470–490 nm, whose amplitudes were four- to sixfold greater than that of wild-

type for equal numbers of molecules (*a* in the figure). These results exclude vinyl formation. The Ser 65→Thr mutant (S65T) was selected for further characterization because it had the longest wavelengths of excitation and emission (490 and 510 nm), which closely resembled those reported for *Renilla* GFP (498 and 508 nm). The crucial post-translational oxidation⁸ to produce the fluorophore from the nascent polypeptide chain proceeded about fourfold more rapidly in S65T than in the wild-type protein (*b* in the figure). This acceleration ameliorates a potentially significant limitation in using GFP as a reporter protein for rapid gene inductions⁹.

Mutations of Ser 65 to Arg, Asn, Asp, Phe, and Trp gave fluorescence intensities well below that of wild type. It remains

unclear exactly how position 65 controls spectral properties or why *Aequorea* chose serine. Nevertheless, the greatly increased brightness and rate of fluorophore generation in mutants such as S65T should make them superior to wild-type *Aequorea* GFP for most experimental uses.

Note added in proof: GFP variants generated by combinatorial mutagenesis of positions 64–69 have excitation peaks near 490 nm, but their amplitudes and the kinetics of fluorophore formation have not been quantified¹².

Roger Heim

Andrew B. Cubitt

Roger Y. Tsien*

Howard Hughes Medical Institute

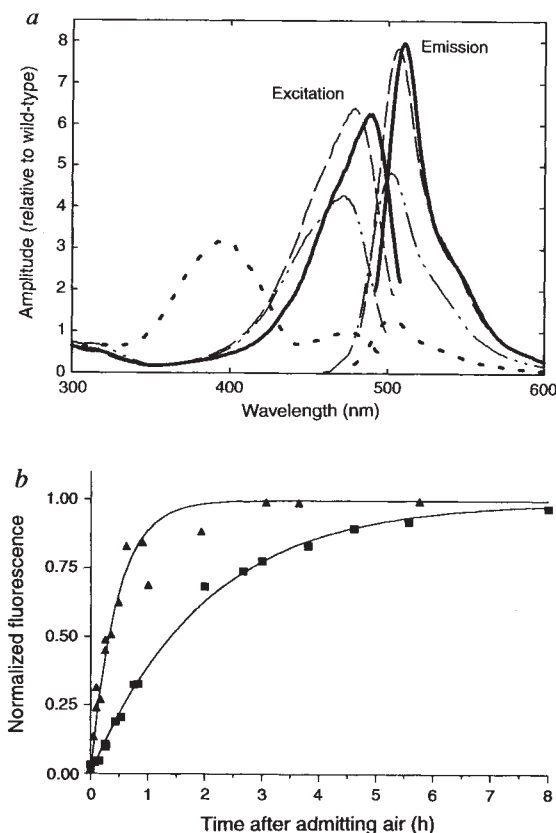
and Department of Pharmacology,

University of California, San Diego, La Jolla, California 92093-0647, USA

*To whom correspondence should be addressed.

- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. *Science* **263**, 802–805 (1994).
- Prasher, D. C. *et al.* *Gene* **111**, 229–233 (1992).
- Wang, S. & Hazelrigg, T. *Nature* **369**, 400–403 (1994).
- Ward, W. W., Cody, C. W., Hart, R. C. & Cormier, M. J. *Photochem. Photobiol.* **31**, 611–615 (1980).
- Ward, W. W. *et al.* *Photochem. Photobiol.* **35**, 803–808 (1982).
- Ward, W. W. in *Bioluminescence and Chemiluminescence* (eds DeLuca, M. A. & McElroy, W. D.) 235–242 (Academic, New York, 1981).
- McCapra, F., Razavi, Z. & Neary, A. P. *JCS chem. Commun.* 790–791 (1988).
- Heim, R., Prasher, D. C. & Tsien, R. Y. *Proc. natn. Acad. Sci. U.S.A.* **91**, 12501–12504 (1994).
- Kunkel, T. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 488–492 (1985).
- Inouye, S. & Tsuji, F. I. *FEBS Lett.* **341**, 277–280 (1994).
- Inouye, S. & Tsuji, F. I. *FEBS Lett.* **351**, 211–214 (1994).
- Delagrè, S., Hawtin, R. E., Silva, C. M., Yang, M. M. & Youvan, D. C. *Bio/Technology* **13**, 151–154 (1995).

Comparison of recombinant wild-type and mutant green fluorescent proteins. *a*, Fluorescence excitation and emission spectra of wild-type protein (—), Ser 65→Ala (---), Ser 65→Cys (—) and Ser 65→Thr (—) mutants. The coding region of *Aequorea* *gfp* cDNA² (gift of D. Prasher) was cloned into the T7 expression vector pRSETB (Invitrogen), giving a polyhistidine-tagged fusion protein expressed in *Escherichia coli*, BL21(DE3)LysS (Novagen). Oligonucleotide-directed mutagenesis at the codon for Ser 65 was performed⁹ on the same construct using the Muta-Gene Phagemid *in vitro* kit version 2 (Bio-Rad). All fusion proteins were purified on nickel-chelate columns (Qiagen). Spectra were obtained from equal concentrations of GFPs controlled by densitometry of denaturing gels stained with Coomassie blue. Excitation spectra were obtained collecting emission at the respective peak wavelengths (508, 503, 507, and 511 nm for wild-type, S65A, S65C and S65T) and were corrected by a quantum counter; emission spectra were likewise measured at the respective excitation peaks (475, 471, 479, and 489 nm) and were corrected using factors from the fluorometer manufacturer. The amplitude of the 475 nm excitation peak of wild-type GFP has been defined as 1.0. The sixfold greater peak amplitude of S65T arises from a 5.5-fold higher extinction coefficient (39,200 M⁻¹cm⁻¹ at 490 nm for S65T compared with 21,000 and 7,150 M⁻¹cm⁻¹ at 395 and 475 nm for wild-type), similar fluorescence quantum yield (0.68 versus 0.77), and slightly narrower emission spectrum. The extinction coefficients and quantum yields reported here for recombinant wild-type protein fused to a polyhistidine tag are in good agreement with literature⁶ values for GFP extracted from *Aequorea* when corrected for the revised molecular mass². Recombinant expression and polyhistidine tagging are known^{1,10,11} not to affect the fluorescence spectra of wild-type GFP. *b*, Rates of autooxidative fluorophore generation in wild-type (■) and S65T (▲) GFP, measured by development of fluorescence after admission of air to *E. coli* cultures anaerobically grown in GasPak pouches (Becton-Dickinson) for 3 days. Air was readmitted while transferring the cells to phosphate-buffered saline containing 8 mM NaN₃ as a metabolic inhibitor. The time course of subsequent fluorescence development measured the final oxidation step in the protein's self-modification to generate its internal fluorophore⁸. Data from two independent runs normalized by their respective asymptotic fluorescence values were pooled for each protein. The smooth curves are exponential curve fits consistent with pseudo-first-order kinetics, with time constants of 2.0 and 0.45 h for wild-type and S65T, respectively. Previously reported time constants for wild-type GFP autooxidation⁸ were rather longer, probably because the protein was held in bacteria for longer periods of anaerobic growth, which seems to slow subsequent oxidation.



Kinetics of protein folding

SIR — Šali *et al.*¹ have attempted to resolve the “Levinthal paradox” of how proteins find their unique native conformations so fast. Although we agree with some of their points, we question others.

First, a model can bear on the Levinthal paradox only if the folding kinetics are run at a temperature low enough for the native state to be more stable than the denatured states. But Šali *et al.* are not studying native conditions: their molecules are mostly denatured. The temperatures they use are so high that equilibrium populations of the native states of many of their “folding sequences” are only 1–5% (ref. 2), and none exceeds 40% (ref. 1). Other model studies³ show that native states can be accessed quickly in certain ranges of denaturing temperatures, but most of the chains will not stay there. If Šali *et al.* could not find native states under folding conditions, they have not completely addressed the Levinthal paradox.

Second, Šali *et al.* state that the “neces-

sary and sufficient condition for a sequence to fold rapidly in the present model is that the native state is a pronounced energy minimum¹² and "the features that depend only on the lower discrete part of the spectrum can be characterized by use of the compact self-avoiding chains alone, neglecting the non-compact conformations"². But this conclusion is contradicted by 11 of the sequences they studied for which lowest-energy conformations are not maximally compact². The true denatured ensemble is generally much larger than the maximally compact ensemble, so neither the first excited state "energy gap" ΔE_{10} , defined by them, nor the temperature, T_x , which they define using only the maximally compact ensemble², is precisely related to true thermodynamic stability. Furthermore, the correlation they observed between their energy gap and folding kinetics is only a weak trend, and the energy-gap condition they use is not sufficient to discriminate between folding and non-folding sequences (see Fig. 7 of ref. 2). In general, folding rate depends on the entire energy landscape³⁻⁶, not just the energy gap in a highly restricted ensemble.

Ultimately, the Levinthal problem is not that a protein has too many degrees of freedom. It is the shape, not the size alone, of the conformational energy landscape that matters^{4-6,9}. Many large land-

scapes have shapes that can be quickly traversed to reach the bottom⁶⁻⁹. Šali *et al.* show that Metropolis Monte Carlo sampling can find the lowest energy of a particular parameterized potential function, but this was already clear from many earlier efforts^{10,11}. The issue, therefore, is whether their potential function is better than earlier models. Baldwin in News and Views¹² has said that Šali *et al.* were using a potential function of the Miyazawa-Jernigan type, picked from the pairwise interactions in the protein database. But, as Šali *et al.* have noted¹, the terms are picked from a random gaussian distribution, not from the databank. Their potential function is not particularly physical, as correlations among contact energies of different pairs of amino-acid residues are neglected. It is unclear whether the potential is any more or less protein-like than any of the potentials used in previous works.

Baldwin¹² describes the work of Šali *et al.* as an important "new view" of protein folding. Naturally, lattice models are useful for addressing general physical principles of protein folding, even though they involve considerable simplification. However, it is clear from many earlier efforts, including some that used comparable lattice simulations, that many of the ideas Baldwin cites as "new" are already in the literature (refs 4, 10, 13-18, and refs therein, and reviewed more recently in refs 3, 5, 6).

Hue Sun Chan

Department of Pharmaceutical Chemistry,
University of California at San Francisco,
California 94143-1204, USA

This note was written with the assistance and concurrence of: Joseph D. Bryngelson, NIH, Bethesda, Maryland 20892, USA; Carlos J. Camacho, Facultad de Física, PUC, Casilla 306, Santiago 22, Chile; Ken A. Dill, Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-1204, USA; José N. Onuchic & Nicholas D. Socci, Department of Physics, University of California at San Diego, La Jolla, California 92093-0319, USA; D. Thirumalai, Institute for Physical Science and Technology, University of Maryland, College Park, Maryland 20742, USA; and Peter G. Wolynes, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801, USA.

KARPLUS *ET AL.* REPLY — Chan *et al.* raise several questions, all of which have simple answers. The object of our study^{1,2} was to examine a large number of sequences and to separate those that fold from those that do not. Consequently, a temperature slightly above T_m the midpoint of the folding transition, was used to speed up the reaction. If folding to the native state were always possible under the simulation conditions, as Chan *et al.* imply there would not have been any non-folding sequences and our computer experiment would have failed. But this is not the case. Further, the same folding kinetics is observed throughout the temperature range where the true native state stability varies from 1 to 40%¹⁹.

A pronounced energy gap between the native and first excited state (equations

(3) and (10) in ref. 1) for the fully compact ensemble, is a necessary and sufficient condition for rapid folding in the model study. It is necessary because no sequence without such a minimum folds to the native state, and is sufficient because all sequences with such a minimum do fold (Fig. 7 of ref. 2). As to the 11 out of 200 sequences that have their minimum outside the fully compact set, none satisfied the energy condition nor did they fold repeatedly either to the lowest fully compact state or to the lowest energy state found by a Monte Carlo simulation. Thus, these sequences confirm and generalize the folding criterion². Further, the use of the energy condition for quantitatively determining the folding rate has been demonstrated²⁰. There is a strong correlation between the results from the fully compact states and the complete set of states (ref. 2 and Fig. 17 therein).

The nature of the configuration space, as well as the number of conformers, is important for the Levinthal paradox^{1,2}. Surfaces can be constructed for which resolution of the paradox is trivial, but this is not true for the 27-mer since only a fraction of sequences fold rapidly. The large size of the configuration space is necessary for the existence of a paradox. The 27-mer model has 10^{16} configurations and requires fewer than 5×10^7 Monte Carlo steps to find the native state. Short oligomers that have been extensively studied on a two-dimensional square lattice^{16,21} may be too small; for example, more Monte Carlo steps (10^5 or more) than there are configurations (4×10^4) were required for folding a 13-mer¹⁶.

The aim of the lattice simulations was to use random interactions so as to determine what differentiates folding from non-folding sequences. The exact choice of parameters was not important, as long as a reasonable set was used. The 27-mer parameters² correspond to the Miyazawa and Jernigan set²² in terms of the magnitude of the interaction energies and their standard deviation.

As to Baldwin's statement in News and Views that we presented a "new view" of protein folding, we agree that some of the concepts in refs 1 and 2 were presaged in earlier work of Go and Abe²³ and of others cited in refs 1 and 2. The 27-mer model studies^{1,2,18} provided the first demonstration that the energy-gap condition and a detailed mechanism for resolving the Levinthal paradox could be found *a posteriori* in computer experiments without having to be introduced explicitly *a priori* to achieve folding²⁴.

M. Karplus

A. Šali

E. Shakhnovich

Department of Chemistry,
Harvard University,
Cambridge, Massachusetts 02138, USA

- Šali, A., Shakhnovich, E. I. & Karplus, M. *Nature* **369**, 248-251 (1994).
- Šali, A., Shakhnovich, E. I. & Karplus, M. *J. molec. Biol.* **235**, 1614-1636 (1994).
- Socci, N. D. & Onuchic, J. N. *J. chem. Phys.* **101**, 1519-1528 (1994).
- Camacho, C. J. & Thirumalai, D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6369-6372 (1993).
- Chan, H. S. & Dill, K. A. *J. chem. Phys.* **100**, 9238-9257 (1994).
- Bryngelson, J. D., Onuchic, J. N., Socci, N. D. & Wolynes, P. G. *Proteins: Struct. Funct. Genet.* (in the press).
- Taketomi, H., Ueda, Y. & Gō, N. *Int. J. Peptide Protein Res.* **7**, 445-459 (1975).
- Zwanzig, R., Szabo, A. & Bagchi, B. *Proc. natn. Acad. Sci. U.S.A.* **89**, 20-22 (1992).
- Dill, K. A. *Curr. Opin. struct. Biol.* **3**, 99-103 (1993).
- Honeycutt, J. D. & Thirumalai, D. *Proc. natn. Acad. Sci. U.S.A.* **87**, 3526-3529 (1990).
- Skolnick, J., Kolinski, A. A. *Rev. phys. Chem.* **40**, 207-235 (1989).
- Baldwin, R. L. *Nature* **369**, 183-184 (1994).
- Bryngelson, J. D. & Wolynes, P. G. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7524-7528 (1987).
- Guo, Z., Thirumalai, D. & Honeycutt, J. D. *J. chem. Phys.* **97**, 525-535 (1992).
- Leopold, P. E., Montal, M. & Onuchic, J. N. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8721-8725 (1992).
- Miller, R. J. *et al. chem. Phys.* **96**, 768-780 (1992).
- Goldstein, R. A., Luthey-Schulten, Z. A. & Wolynes, P. G. *Proc. natn. Acad. Sci. U.S.A.* **89**, 9029-9033 (1992).
- Shakhnovich, E., Farztdinov, G., Gutin, A. M. & Karplus, M. *Phys. Rev. Lett.* **67**, 1665-1668 (1991).
- Karplus, M., Caffish, A., Šali, A. & Shakhnovich, E. in *Modelling of Biomolecular Structures and Mechanisms* (eds Pullman, A. *et al.*) 69-84 (Kluwer Academic, Dordrecht, 1994).
- Abkevich, V. I., Gutin, A. M. & Shakhnovich, E. *J. chem. Phys.* **101**, 6052-6062 (1994).
- Camacho, C. J. & Thirumalai, D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6369-6372 (1993).
- Miyazawa, S. & Jernigan, R. L. *Macromolecules* **18**, 534-552 (1985).
- Go, N. & Abe, H. *Adv. Biophysics* **18**, 149-164 (1984).
- Karplus, M. & Šali, A. *Curr. Opin. struct. Biol.* **5**, 58-73 (1995).

Hot white dwarfs

SIR — THE discovery of a very hot hydrogen-rich white dwarf — RE1738+665 — in the Rosat survey (ref. 1) has generated much excitement (see the discussion in News and Views²). Although undoubtedly interesting, claims that it is a missing link are greatly exaggerated. Research over the past two years has shown that there are a substantial number of white dwarfs that help plug the so-called generation gap — both those with and without ancient planetary nebulae. The lack of a clear division between the two groups is well illustrated by RE1738+665 itself: although it has been regarded as an isolated white dwarf, it does seem to have a planetary nebula associated with it³. There remain other problems with the current understanding of hot white dwarf evolution, but RE1738+665 is just one of several pieces of evidence showing that the elegant single-channel evolutionary model is long outdated.

A key feature of the single-channel model was that it could explain why H-rich white dwarfs could be found only in two separate groups. These were the low-gravity central stars ($T_{\text{eff}} < 80,000$ K, surface gravity $\log g < 5.5$; ref. 4) and the high-gravity white dwarfs which had cooled back below 70,000 K (ref. 5). The hottest known white dwarfs (with $70,000 \text{ K} < T_{\text{eff}} < 170,000 \text{ K}$ and surface gravity $\log g \sim 7$, for example PG1159-035; ref. 6) were all devoid of hydrogen. This has now changed. In their study of H-rich white dwarfs found as central stars of planetary nebulae, Napiwotzki and Schönberner^{7,8} found 11 of 15 with $T_{\text{eff}} > 70,000$ K, including NGC7293, the central star of the closest known planetary nebula, Sh2-216, and WDHS1, which may even be as hot as 200,000 K. The parameters derived for three of these stars have been independently confirmed^{9,10}. Higher temperatures are also being reported for known isolated white dwarfs, including PG1342+444 at 79,000 K, PG0939+262 (Ton320) at 77,000 K, and the remarkable PG0948+534, with

$T_{\text{eff}} = 130,000$ K and $\log g = 7.4$ (ref. 9).

The uniqueness of RE1738+665 lies in its being an EUV/soft X-ray source. It was a striking result of the all-sky survey performed by Rosat that essentially no H-rich white dwarfs above about 65,000 K were found¹¹⁻¹³. Furthermore, those detected close to this upper limit have very steep short-wavelength cutoffs, together with ultraviolet spectra that are littered with many weak heavy-element lines¹². Above this temperature, radiation pressure acting on the heavy elements appears to be sufficient to counteract the high gravities; their presence in the photosphere blocks out the short-wavelength emission. The sole exception to this is RE1738+665, which at 88,000 K is by far the hottest H-rich white dwarf detected by Rosat. The reason is probably that it has a rather high mass. The value derived by Barstow *et al.*¹ of $0.62 M_{\odot}$ is likely to be incorrect since they use evolutionary models that were designed for understanding old, cool white dwarfs¹⁴, which do not take into account the prior evolution from the AGB, and which is nec-

essary for the hot stars¹⁵. Using the more accurate tracks in ref. 15 leads to a mass $\sim 0.7 M_{\odot}$ — high compared with the white-dwarf mass distribution, where 75% lie between 0.45 and $0.65 M_{\odot}$ (ref. 16). This higher gravity means that fewer heavy elements can be levitated to the surface layers, which increases the transparency to EUV/soft X-ray emission. High masses have also been reported for the pure-H central stars, EGB1 and WDHS1 (ref. 8), which Barstow *et al.*¹ refer to as possible progenitors of RE1738+665. Subdividing the H-rich objects on the basis of presence or absence of He is meaningful only insofar as it highlights the importance of the mass of the remnant. RE1738+665 is a fascinating object, but its importance to understanding white-dwarf evolution should not be overstated.

Richard W. Tweedy
Steward Observatory,
University of Arizona,
Tucson,
Arizona 85721,
USA

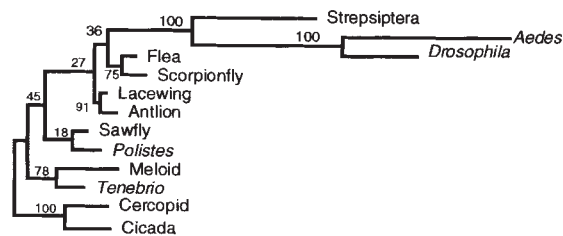
Do long branches attract flies?

SIR — We dispute Whiting and Wheeler¹ statement that 18S ribosomal DNA sequence data support the Strepsiptera as sister-group to the Diptera (flies). Diptera and Strepsiptera group together in phylogenetic analyses, but we interpret this as an artefact resulting from the high substitution rates in their 18S rDNA sequences. Long-branch lineages — lineages with high substitution levels — will cluster together with strong statistical support^{2,3}.

We used complete 18S rDNA insect sequences (Collembola, GenBank accession number Z26765; Hemiptera, U06478 and U06480; beetles, X07801 and X77786; Strepsiptera, X77784; *Polistes* wasp, X77785; *Aedes* mosquito, X57172; and *Drosophila*, M21017) and aligned them with partial sequences (~1,090 nucleotides: several Hymenoptera, two Neuroptera, a flea and a scorpionfly)⁴. Nucleotide substitution rates in 18S rDNA of flies and Strepsiptera were dramatically higher than in the other holometabolous insects (see figure), but these substitutions were not homologous with each other. Whether or not the strepsipteran and flies group with the scorpionfly and flea (which are close to the flies⁵) depends on the taxa and portion of the gene included in the analysis.

Phylogenetic placement of the Strepsiptera is an old problem which many entomologists are working to resolve⁵⁻⁷. Whiting and Wheeler¹ may be right —

flies and strepsipterans might be close relatives. In our analyses the 18S rDNA data provide no evidence to support this conclusion. However, because Whiting and Wheeler have not made their data available, it has not been possible to examine



Bootstrap consensus phylogram from parsimony (PAUP 3.1, 2,000 replicates, 889 steps) showing the long branches of the Diptera and Strepsiptera.

the opposing evidence. Instead, the grouping of the Strepsiptera and Diptera in the 18S rDNA tree appears to be an excellent example of long-branch attraction resulting from the violation of the molecular clock for this gene.

David Carmean, Bernard J. Crespi
Department of Biological Sciences,
Simon Fraser University, Burnaby,
British Columbia V5A 1S6, Canada

1. Whiting, M. F. & Wheeler, W. C. *Nature* **368**, 696 (1994).
2. Hillis, D. M. *et al.* *Nature* **369**, 363-364 (1994).
3. Kühner, M. K. & Felsenstein, J. *Molec. Biol. Evol.* **11**, 459-468 (1994).
4. Carmean, D., Kimsey, L. S. & Berbee, M. L. *Molec. Phylogenet. Evol.* **1**, 270-278 (1992).
5. Kristensen, N. P. in *Insects of Australia* 2nd edn (ed. CSIRO) 125-140 (Melbourne Univ. Press, 1991).
6. Afzelius, B. A. & Dallai, R. J. *Morph.* **219**, 15-20 (1994).
7. Kathirithamby, J., Carcupino, M. & Mazzini, M. *Int. J. Insect Morphol. Embryol.* **22**, 459-470 (1993).

1. Barstow, M. A. *et al.* *Mon. Not. R. astr. Soc.* **271**, 175-182 (1994).
2. Shipman, H. L. *Nature* **372**, 318-319 (1994).
3. Tweedy, R. W. & Kwitter, K. B. *Astrophys. J.* **433**, L93-L96 (1994).
4. Mendez, R. H. *et al.* *Astr. Astrophys.* **190**, 113-136 (1988).
5. Holberg, J. B. *et al.* *IAU Colloq.* **114**, 188-193 (1989).
6. Werner, K., Heber, U. & Hunger, K. *Astr. Astrophys.* **244**, 437-461 (1991).
7. Napiwotzki, R. & Schönberner, D. in *White Dwarfs: Advances in Theory and Observation* (ed. Barstow, M. A.) 99-105 (Kluwer, Dordrecht, 1993).
8. Napiwotzki, R. thesis, Univ. Kiel (1993).
9. Bergeron, P. *et al.* *Astrophys. J.* **432**, 305-325 (1994).
10. Liebert, J. W., Bergeron, P. & Tweedy, R. W. *Astrophys. J.* **424**, 817-822 (1994).
11. Barstow, M. A. *et al.* *Mon. Not. R. astr. Soc.* **264**, 16-34 (1993).
12. Holberg, J. B. *et al.* *Astrophys. J.* **416**, 806-819 (1993).
13. Fleming, T. A. *Adv. Space Res.* **13**, (12)7-13 (1993).
14. Wood, M. A. *Astrophys. J.* **386**, 539-561 (1992).
15. Böcker, T. & Schönberner, D. *Astr. Astrophys.* **240**, L11-L13 (1990).
16. Bergeron, P. *et al.* *Astrophys. J.* **394**, 228-247 (1992).

Grandiose conceits

John Morton

Making Monsters: False Memory, Psychotherapy and Sexual Hysteria. By Richard Ofshe and Ethan Watters. Scribner's: 1994. Pp. 340. \$23.

RICHARD Ofshe is a professor of social psychology at the University of California, Berkeley, and Ethan Watters is a freelance magazine writer. Ofshe, a specialist in cults, became interested in 'false memory' when he was asked by US prosecution lawyers to assist in the investigation of a case of alleged satanic ritual abuse in which the possibility of false memories had been raised. While interviewing Paul Ingram, the accused man, Ofshe began to suspect that Ingram was not confessing to crimes that he had actually committed but was instead inducing himself into a trance and imagining crimes that had been suggested to him during the investigation. To confirm his suspicion, Ofshe conducted an experiment. He invented a completely new allegation and asked Ingram whether he remembered the incident. Initially, Ingram said that he had no memory of the events described, but later he not only claimed to remember them but also substantially elaborated the story. Later still, despite having his elaborated story challenged, Ingram could not be shaken from his belief that the events contained in this pseudo-memory had in fact taken place. Ofshe had demonstrated that Ingram had created a false memory, but Ingram remained utterly convinced of the truth of the crimes to which he had confessed and is now serving a 20-year prison sentence. As a result, Ofshe became increasingly concerned about the proliferation of accusations of sexual abuse and of the predicament of those who had been falsely accused.

Ofshe has now become a campaigner on behalf of these individuals, and is a leading adviser to the False Memory Syndrome Foundation in the United States. More than 15,000 people are said to have registered with this organization in the past few years. Many are parents who maintain that they have been falsely accused. There are 550 families now registered with the equivalent organization in the United Kingdom, the British False Memory Society. The position of these parents' associations is not only that the parents have been falsely accused but that the memories of abuse have been implanted in the minds of the alleged victim during a form of therapy that the authors call 'recovered-memory therapy'. Ofshe and Watters tell several persuasive stories to illustrate the adverse effects of this therapy and paint an alarming picture of widespread therapeutic malpractice in the United States. Their tales of the changes

in the beliefs of accusing children and of the resulting destruction of families are moving. In most of the accounts, the villains are clearly the 'therapists' who believe that a wide variety of psychological problems — depression, eating disorders, sexual dysfunction, low self-esteem, difficulty maintaining a relationship, suicidal or self-destructive thoughts — are invariably indicators of sexual abuse in childhood. The therapists are often



depicted as determined to demonstrate to a client that he or she can retrieve memories of having been sexually abused and if necessary they will persevere for years to achieve this end. According to the authors, hypnotic regression techniques are commonly used.

These stories form the background to one of the central themes of the authors, an attack on recovered-memory therapy, a phenomenon that they say is making 'chilling' inroads into US culture (although serious doubts have been cast on the accuracy of one of their stories in a recent review of the book in the *Los Angeles Times*). They paint an alarming picture of the way in which the whole topic of recovered memories is becoming commercialized and exploited. For example, recovered-memory patients and experts on the subject have become staples of talk shows, and popular books on the topic have sold millions of copies. Insurance companies are said to have paid out hundreds of millions of dollars for 'memory therapy' over the past dozen years. Specialist 'dissociation clinics' have sprung up, admitting women for intensive treatment (paid for by private medical insurance). Here, the women's condition deteriorates as their beliefs about their past become more florid, but when their medical insurance limit is reached, they are shown the

door. These changes have even affected the legal system and become a lucrative area for lawyers seeking to make large sums of money. About half the states have enacted changes in their statutes of limitations to allow recently recovered memories of abuse to become the basis for civil lawsuits with heavy legal costs.

According to Ofshe and Watters, those who claim to have recovered memories of abuse that were previously forgotten are labouring under a tragic misapprehension. How, they ask, could such events have been forgotten? The mechanism of forgetting in these cases was supposed to be 'repression'. This is characterized by Ofshe and Watters in the following way:

Not only are the memories said to be hidden, but they lie frozen in pristine form, awaiting a time when the person is emotionally prepared to remember. The final magic of this new robust repression mechanism is the belief among recovered-memory therapists that these traumatic events can be 're-lived' during therapy. So real are these repressed memories said to be that therapists claim clients reexperience the event as if it were happening at the moment.

Robust repression of this nature becomes the authors' target for attack along with recovered-memory therapy. The concept of repression as described by Freud was never more than a "grandiose conceit" and is now, the authors maintain, completely discredited, and with it all forms of therapy that involve attempts to create a meaning for symptoms in the present by exploring the patient's experience of the past. As far as the authors are concerned, there are no grounds whatsoever, either clinical or academic, to justify recovered-memory therapy.

What is astonishing, perhaps, is that so many people are happy to postulate mechanisms of memory loss without having a theory of memory itself. With only folk theory of memory, we should expect folk accounts of memory loss. Ofshe and Watters have no coherent theory of memory themselves, but are happy to attack the folk accounts. They write as if they have the full authority of science behind them. The jacket of the book calls it "ground-breaking science", but it is polemic, not science. They poke fun at people at the practical end of the controversy for their incoherent and inconsistent notions about the possible mechanism of amnesia. These people, including psychiatrists and academic psychotherapists, may indeed have some strange ideas about the properties of memory. But Ofshe and Watters are in no better position. There is no description or discussion of amnesia in any of its forms, either organic or psychogenic (fugal states), although these are well enough documented. They say little about what is known about event memory and nothing about the range of current psychological theories, some fairly computational, that could provide alternative accounts of amnesia. Most leading workers in the field

agree that there can be amnesia for traumatic events. The questions and disagreements relate to the natural frequency of amnesia, its circumstances and its mechanism. We should not reject phenomena simply because there is no agreed explanation.

Ofshe and Watters include some spirited calls for action against recovered-memory therapy. They suggest, however, that trust should not be placed in the large professional organizations in the mental-health field, which "function less as regulatory bodies insuring the safety of the patients than they do guilds protecting and promoting their membership". Sadly, the authors fail to come up with alternative suggestions as to how therapeutic good practice should be protected and developed, and how afflicted families might be helped. This is an important opportunity missed.

In the recent British Psychological Society report on recovered memories, recovered-memory therapy was not attacked because there was no evidence that it was a threat in the United Kingdom. Furthermore, the professional organizations concerned with training and accrediting therapists seem to be taking a responsible attitude. It is generally agreed that everything possible should be done to prevent the growth in the United Kingdom of problems such as those described by Ofshe and Watters. The report therefore goes to some lengths to provide recommendations that will enable therapists to take practical steps to develop good practice in line with the best scientific information about how memory works.

Ofshe and Watters write well and may be on target for their own culture, but this is a book that inflames rather than instructs. A better polemic, because it is less pretentious and more informative, is *Victims of Memory* (Upper Access, 1994) by Mark Pendergrast, and less polemical and more academic is *The Myth of Repressed Memory* (St Martin's, 1994) by Elizabeth Loftus and Katherine Ketcham. All three books warn against the excesses to be found in the United States, and other countries should take this warning seriously. None of them is a sober work of science, and the authoritative book has still to be published. Many of these issues can be thought about scientifically, and the August 1994 special issue of the journal *Applied Cognitive Psychology* is a good illustration, with a principal article by two Canadians, Stephen Lindsay and Don Read, and half-a-dozen commentaries. □

John Morton is in the Medical Research Council Cognitive Development Unit, 4 Tavistock Street, London WC1H 0AH, UK. He chaired the British Psychological Society's working party on recovered memories.

Minds in the unmaking

Hugh Freeman

House of Cards: Psychology and Psychotherapy Built on Myth. By Robyn M. Dawes. Free Press: 1994. Pp. 338. \$22.

THE casting down of idols following an excess of uncritical enthusiasm is a specially American phenomenon. Among these household gods, psychological treatment ultimately derived from the work of Freud has had a prominent place for much of this century. It also represents an enormous national investment. Robyn M. Dawes, a professor of social and decision sciences at Carnegie-Mellon University in Pittsburgh, Pennsylvania, reveals here that the American Psychological Association (APA) has nearly 70,000 members, of whom 40,000 are engaged in psychotherapy or counselling. This is in addition to around 35,000 psychiatrists — mostly doing the same kind of work — and innumerable social workers, counsellors and others of diminishing respectability. Granted that this is a very affluent society — at least the part that J. K. Galbraith described as the "contented majority" — some kind of quality assessment of the psychological help it uses seems overdue.

Dawes is clearly something of a thorn in the APA's flesh, condemning its policies on training, professional licensing, fees and testimony in courts. The APA will support psychologists, he says, "in doing virtually anything at all that appeals to their 'clinical intuition', as if there were no scientific knowledge". What is more, this situation has been getting worse, as the profession's commitment to using research findings as a basis for practice has steadily eroded. 'Graduates' in psychology may now have had hardly any scientific training.

Not only are these procedures unrelated to any well validated scientific theory, but the lack of systematic feedback means that practitioners have no reliable way of knowing whether they are making any real difference to their patients, or whether their predictions about them are right. Many of the people in therapy may feel better after a certain time, but Dawes points out that this can be simply a 'regression' effect whereby a less likely state (very unhappy and distressed) becomes a more likely one (average mood). In any case, those who actively seek help may have greater personality resources on average than those who don't, so that their prognosis is better, while the process of obtaining help may be therapeutic in itself.

The only validated way of testing these

interventions is by some form of randomized controlled trial (which the author misnames), but to do this with psychotherapy is a methodological nightmare. Nevertheless, a great deal of research effort has gone into the attempt, although its literature is not adequately described here. Dawes accepts that psychotherapy can be of value, but regards existing training and licensing arrangements in the United States as simply the blind leading the blind: without adequate scientific understanding and systematic feedback, the quality of service can never be improved. Worst of all, in Dawes's view, is the fact that techniques that research has clearly shown to be invalid — particularly the Rorschach projective test — are still widely used by US psychologists.

The author's most palpable hit is at the activities of mental-health 'experts' in court cases about, for example, alleged child abuse. This kind of evidence has profound effects on people's lives, breaking up marriages and families, putting men in prison for years and, through the 'recovery' of nonexistent memories, leaving children with the conviction that they were once abused. Because of the lack of objective evidence in these cases, courts are persuaded by the claims of practitioners that, on the basis of their experience, they 'know' what really took place. But as Dawes points out, tests of feelings and attitudes tell us nothing about what actually happened, while the 'symptoms' claimed to indicate abuse include virtually every kind of behaviour that may cause concern to parents. A further escalation of the process, not mentioned here, is that the alleged victims may then be diagnosed as having 'multiple personality disorder' — a condition largely induced by the therapists themselves.

This kind of rigorous common sense could have a healthy effect on professional practice, particularly in the United States, and Dawes says that it "often has the same implications" for psychiatry and social work as it has for clinical psychology. But that is not really fair to biologically based psychiatry, to practical social work or to psychological techniques derived from learning theory. In all these areas, he shows himself to be seriously out of his depth. To describe current psychotropic drugs, for instance, as "narcoleptics" shows a complete misunderstanding of their action, and although it is possible that they may have "long-term deleterious effects", he does not consider the undoubtedly harmful effects for many patients of *not* using them.

What is most worrying, though, is the cultural insularity of this work. Out of several hundred references, less than a dozen are from outside the United States; only four are British (three of them very old). Most of Dawes's concerns about

psychotherapy were in fact first raised by H. J. Eysenck in 1952, yet that name appears here only as quoting a US author. This seems to indicate something else that is seriously wrong with US psychology. □

Hugh L. Freeman, formerly editor of the British Journal of Psychiatry, is at 21 Montagu Square, London W1H 1RE, UK.

Codebreaking

David Knight

Gehennical Fire: The Lives of George Starkey, an American Alchemist in the Scientific Revolution. By William R. Newman. Harvard University Press: 1994. Pp. 348. \$49.95, £39.95.

LIKE Chaucer and Ben Jonson, we associate alchemy with rogues and confidence men; or perhaps with "the lunatic, the lover, and the poet. . . of imagination all compact". We tend to believe that charlatans, the self-deceived or those in search of tropes and images involving sexual union and perfection were the kinds who took up with alchemy, but that to the serious science of chemistry it was but a step-mother, perhaps delaying the 'Chemical Revolution' so that Antoine Lavoisier's *Elements of Chemistry* came a whole century after Isaac Newton's *Principia*. And serious alchemy in America, among sober Puritans with their work ethic, would seem quite incongruous.

William Newman shows how mistaken such preconceptions are. To recover the worldview of alchemists is no easy matter and demands the disciplined use of the historical imagination: his style, confident, spirited and ironic, and his evident knowledge and enthusiasm carry us back into another world. It is not entirely a brave new world, for students of Newton have for many years been foraging among his copious alchemical manuscripts, trying to establish which are original writings and which are copies; and Robert Boyle's interest in alchemy is also being explored. The texts, operating on different levels, need interpretation; written both to impart and to withhold information, they have always needed decoding. To this hermeneutics, Newman is an excellent guide.

His hero, if that is the word, is George Stirk or Starkey. Born in Bermuda of Scottish parents in 1628, he matriculated at Harvard in 1643 and graduated as a bachelor of arts in 1646. He was one of a class of only four, and at Harvard would have encountered what might seem to us a curious hybrid: a belief that matter was made up of corpuscles, within an Aristotelian framework. It is part of Newman's

thesis that early modern atomism is not a feature only of the 'mechanical worldview' in the tradition of Galileo and René Descartes. Starkey also developed his interest in the generation of insects; that is, of nature's growth towards perfection. Alchemy, fusing chemical, prophetic and millenarian traditions, was centre-stage at this time.

Moving to London, Starkey entered Samuel Hartlib's circle associated with hopes for practical science, and in 1651 he



"Alchemists at Work" from Philip Ulstadt's *De Secretis Naturae* (1544).

made contact with Boyle. Practising medicine but pursuing alchemy as the way to truth, and boozing, he found himself constantly short of money and was imprisoned for debt; alchemists were also under suspicion of coining. He invented an *alter ego*, Eirenaeus Philalethes, a cosmopolitan sage with whom he claimed to be in privileged contact and whose writings he published. His alchemy derived from a mediaeval corpuscular tradition associated with writings attributed to "Geber" and drawing on "Ramon Lull", Bernard of Trier, Paracelsus and especially J. B. Van Helmont.

Newman penetrates the deliberate obscurity and obfuscation of alchemical texts, seeing them as undoubtedly chemical and decoding the recipes within. Using contemporary authors as guides, he shows the similarities between Starkey's alchemy and his medical chemistry. (Starkey was a great supporter of chemical remedies against the orthodox Galenists.) He illuminates Starkey's search for the alchemical, philosophical mercury or universal solvent; and describes his fascination with antimony and its crystalline star, and his

belief in shells of corpuscles that made up the various metals. Newman shows how this 'paradigm' differs from that of Starkey's contemporary Thomas Vaughan, who confusingly wrote as Eugenius Philalethes and in whose system, building on that of Cornelius Agrippa, the element Earth is primary.

Starkey's life reached its climax in the plague of 1665, when the physician was unable to save himself and died in the course of his duties. He seems to have fallen between two stools: he was neither a respected physician nor a quack committed to selling his remedies through extensive advertising. Moving on the fringe of the respectable world associated with the Royal Society, he never became a pillar of society. Curiously, he was then seen as a bad disciple of his fictive Philalethes, whose reputation long outlasted Starkey's and whose works were avidly studied by Newton — Newton's theory of matter does seem to show connections with Starkey's.

Newman shows how studying an obscure and ambiguous figure can bring the science of a period to life. And he shows that alchemy can be studied like more mainstream science, given the effort required to penetrate its language, verbal and visual. Because his remedies were used by Boyle and his writings studied by Boyle, G. W. Leibniz and Georg Stahl as well as by Newton, Starkey was clearly not an insignificant figure. He makes us think again about the 'Scientific Revolution'. □

David Knight is in the Department of Philosophy, University of Durham, 50 Old Elvet, Durham DH1 3HN, UK.

Integrative animal behaviour

Gene Robinson

Behavioral Mechanisms in Evolutionary Ecology. Edited by Leslie A. Real. University of Chicago Press: 1994. Pp. 469. \$80, £63.95 (hbk); \$29.95, £23.95 (pbk).

In 1975, E. O. Wilson predicted in his masterly *Sociobiology* that animal behaviour would split into two separate disciplines by the year 2000, one devoted to mechanisms and the other to the evolution of behaviour. Trends since then support this prediction. But as we approach the millennium there are signs of a new *rapprochement* in animal behaviour. Some who ask 'how?' are increasingly also asking 'why?', and vice versa. Even federal granting agencies are getting into the act — several institutes at the National Institutes of Health have developed a pro-

gramme in "Comparative Approaches to Brain and Behavior" that weds mechanism and evolution.

Nowhere is this new approach better explicated than in *Behavioral Mechanisms in Evolutionary Ecology*. Arising from a symposium organized by Leslie Real for the American Society of Naturalists in 1992, the book has been expanded to include 19 contributions that provide broad coverage of many important topics in animal behaviour. It begins with an introductory chapter by Real, who brilliantly sketches the rationale for, and outlines of, a more unified approach to animal behaviour. The first part of the book, on "psychological and cognitive foundations", includes chapters by Kamil, Krebs and Inman, and Real, which describe how cognitive and ecological perspectives can be integrated, especially in the context of foraging. Kamil's chapter includes a thoughtful assessment of the traditional psychological approach to learning and one of the clearest expositions of the 'cognitive approach' I have read. Also in this section Dyer presents an illuminating analysis of insect orientation behaviour from a cognitive perspective.

The second part, on "communication", includes a chapter by Ryan on mechanisms of sexual selection. Ryan interweaves neuroethology and evolutionary biology and extends his "sensory exploitation" hypothesis to explain the evolution of female choice in frogs. This chapter illustrates particularly well the way in which sophisticated mechanistic analyses can contribute to important issues in behavioural ecology.

The third part, on "neural, developmental, and genetic processes", includes two chapters on bird-song development: Arthur Arnold's review of diversity in endocrine and neural control in passerines, and West, King and Freeberg's self-critical and perceptive analysis of the role of the environment in cowbirds. There are also two chapters on constraints on behavioural evolution: Stevan Arnold's quantitative genetic analysis, and Singer's case study of the evolution of diet breadth in checkerspot butterflies.

In the fourth part, on "hormonal processes", Ketterson and Val Nolen probe the mechanistic basis of life-history evolution with clever "phenotypic engineering" experiments in which they treat dark-eyed juncos with testosterone implants and then determine the consequences for fitness of variation in hormonally mediated behavioural traits. Zuk advocates a new field of "behavioural immunology" that extends psychoneuroimmunology to deal with questions of behavioural ecology. Although focusing on vertebrates, she mentions that recent findings suggest intriguing similarities between vertebrate and insect

immune systems.

The final part, on "the social context of behavior", is exclusively on primates and social insects. Seyfarth and Cheney have two goals: to infer the cognitive abilities required for primate social life and to explore ecological and social factors that select for them. It is a pleasing blend of convincing data and provocative speculation. Two chapters address a central question in insect sociobiology: how the behaviour of thousands of individual members is integrated into a smoothly running colony. Both are examples of a productive and increasingly popular refinement of the venerable 'superorganism' approach, in which a heuristic metaphor is invoked to help analyse a specific feature of colony biology. Gordon likens an ant colony to a neural network in order to guide novel empirical work on the way in which individual ants gain information on changing colony conditions. Franks and Partridge compare the predatory behaviour of army ant colonies not only to that of myxobacteria and cellular slime moulds, but to a human social construct — "Lanchester's theory of combat". This theory guided Nelson to decisive victory in the battle of Trafalgar in 1805, and it also helps Franks and Partridge to paint a coherent picture of how worker size, colony population size and self-organization contribute to ergonomic efficiency.

The book is nicely put together. Most chapters are well written, contain excellent introductions for nonspecialists and provide comprehensive reviews of the literature. Genetic themes are not as fully covered as I would have liked, and it is surprising that a chapter on kin recognition is not included, given that this phenomenon has always been investigated from a variety of perspectives. Cellular analyses of behaviour are presented only for bird song, and molecular studies are absent. This probably reflects the fact that most behavioural studies at these two levels do not yet involve a strong ecological component.

This is an important collection because it shows the promise of a more integrated approach to animal behaviour. But only a few authors in fact achieve a sophisticated blend of mechanism and evolution; most of the chapters treat one or the other superficially, and some could just have easily appeared in a book on behavioural ecology that does not purport to be integrative. Nevertheless, the volume paints a reasonable picture of current knowledge and reveals many wonderful opportunities for future research. If they are seized, the future of animal behaviour will be bright. □

Gene E. Robinson is in the Department of Entomology, University of Illinois, Urbana, Illinois 61801, USA.

The measure of the world

C. W. Kilmister

Poetry of the Universe: A Mathematical Exploration of the Cosmos. By Robert Osserman. Doubleday: 1995. Pp. 210. \$18.95.

JAMES L. Adams is recorded as asking the author: "How is it that mathematics is such a beautiful subject, yet students can go through four years of college taking many math courses and never find out?" They sought to rectify this with a taught course, and here is part of it. The aim differs subtly from that of popular science and so does the rather elegant result. The atmosphere is leisurely and there is more emphasis on the integration of the history of ideas in mathematics with the surrounding culture than on simplified expositions. (A slightly whimsical view of Euler, Gauss and Riemann as reflecting Bach, Beethoven and Brahms, their near-contemporaries, fits the first two pairs better than the third.)

The chosen storyline is the development of the measurement of "the world" — a term with widening connotation as the story develops. Pythagoras and Euclid lead quickly into Eratosthenes' ingenious and remarkably accurate determination of the circumference of the Earth. Map projections then bring one effortlessly to Gauss, the notion of curvature, the figure of the Earth and non-Euclidean geometry. The smooth development breaks off a little after Riemann. Maxwell, the expanding Universe and Einstein are brought in to illuminate what follows from Riemann's ideas, and the concluding modern cosmology involves topology and chaos.

Only in the final chapters is there material not to be found in many other popular accounts of mathematics, but this book never forgets to live up to its chosen title. The mathematical demands on the reader are virtually nil, but there are 25 pages of notes at the back, often very informative and some involving elementary mathematics. I particularly admired the following one-sentence definition: "The integral calculus is a mathematical technique for evaluating the cumulative effect of a continually changing quantity." □

C. W. Kilmister, formerly in the Department of Mathematics, King's College London, is at Red Tiles Cottage, High Street, Barcombe, Lewes BN8 5DH, UK.

■ In the Beginning: The Birth of the Living Universe by John Gribbin is now published in paperback by Back Bay Books (Little, Brown) at \$12.95.

Pathway of processive ATP hydrolysis by kinesin

Susan P. Gilbert^{*†}, Martin R. Webb[‡], Martin Brune[‡] & Kenneth A. Johnson^{*§}

^{*} Department of Biochemistry and Molecular Biology, 106 Althouse Laboratory, Pennsylvania State University, University Park, Pennsylvania 16802, USA

[‡] National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Direct measurement of the kinetics of kinesin dissociation from microtubules, the release of phosphate and ADP from kinesin, and rebinding of kinesin to the microtubule have defined the mechanism for the kinesin ATPase cycle. The processivity of ATP hydrolysis is ten molecules per site at low salt concentration but is reduced to one ATP per site at higher salt concentration. Kinesin dissociates from the microtubule after ATP hydrolysis. This step is rate-limiting. The subsequent rebinding of kinesin · ADP to the microtubule is fast, so kinesin spends only a small fraction of its duty cycle in the dissociated state. These results provide an explanation for the motility differences between skeletal myosin and kinesin.

KINESIN, a microtubule-activated ATPase, functions as a cytoplasmic motor to drive organelle translocation toward the plus ends of microtubules^{1–3}. The principles governing the conversion of chemical energy from ATP hydrolysis to force production for the sliding of kinesin along microtubules may be similar to those for actomyosin and the axonemal dynein-microtubule ATPases^{4,5}. However, the motility of single motor molecules *in vitro* suggests that mechanochemical coupling for kinesin must be somewhat different from actomyosin. For example, a single molecule of kinesin (2 heads) will promote translocation for several micrometres and at maximal rates⁶. In contrast, multiple skeletal myosin molecules are required for directed movement along an actin filament and the velocity increases as the number of myosin molecules is increased⁷. In addition, the non-hydrolysable ATP analogue AMP-PMP (β , γ -imidoadenosine 5'-triphosphate) causes dissociation of the actomyosin and microtubule-dynein complexes, but promotes stabilization of the microtubule-kinesin complex^{5,8}.

Here we describe mechanistic studies of the kinetics of individual steps in the ATPase cycle of the kinesin ATPase to explain the interactions of kinesin with the microtubule lattice responsible for movement. We have used the *Drosophila* kinesin motor domain expressed in *Escherichia coli*^{9–11}. This protein, designated K401 and containing the N-terminal 401 amino acids, is a fully active, homogeneous preparation with the kinetic and structural properties expected of a native kinesin^{9–11}. It has a very low ATPase activity in the absence of microtubules which is limited by the rate of ADP release ($\sim 0.01 \text{ s}^{-1}$). In the presence of microtubules, the steady-state rate increases to a maximum of $20 \pm 2 \text{ s}^{-1}$. Furthermore, K401 is a dimer under our experimental conditions (J. J. Correia, S.P.G., M. L. Moyer and K.A.J., manuscript submitted).

Chemical quench-flow experiments¹¹ established the rate of ATP hydrolysis at the active site to be significantly faster (100 s^{-1}) than steady-state turnover; therefore, the rate-limiting step of the microtubule-activated ATPase must occur after ATP hydrolysis. These results were initially interpreted to suggest that ADP product release from the microtubule-kinesin · ADP (M-K · ADP) complex is rate-limiting, analogous to the microtubule-dynein ATPase¹² and actomyosin⁴. But our stopped-flow results reported here establish a mechanochemical coupling

mechanism for kinesin distinct from either actomyosin or the microtubule-dynein ATPases. These studies show that ATP binding and ATP hydrolysis occur before dissociation of kinesin from the microtubule. Accordingly, kinesin can remain associated with the microtubule through multiple rounds of hydrolysis because it spends only a small fraction of its 50-ms cycle time dissociated from the microtubule. In addition, kinetic evidence is presented for sequential release of the kinesin heads from microtubules, suggestive of cooperativity which may contribute to the observed processivity in the motility and kinetic experiments. Direct evidence for processive ATP hydrolysis is presented and this is shown to be dependent on salt concentration.

K401 dissociation

Previous chemical quench-flow experiments have defined the kinetics of ATP binding and ATP hydrolysis by the M-K complex¹¹. ATP binding is rapid ($2 \mu\text{M}^{-1} \text{ s}^{-1}$, $k_{\text{off,ATP}} = 200 \text{ s}^{-1}$) but weak, with an apparent $K_{\text{d,ATP}} = 100 \mu\text{M}$, and ATP hydrolysis at the active site is $\sim 100 \text{ s}^{-1}$ (Fig. 1, scheme 1). To determine whether ATP binding induces kinesin dissociation from the microtubule before or after ATP hydrolysis, we measured K401 dissociation from the microtubule directly by stopped-flow turbidity measurements.

Figure 2a shows the time course of K401 dissociation after mixing the M-K401 complex with 1 mM Mg-ATP plus 100 mM KCl. The observed rate of the fast phase is a function of ATP concentration (Fig. 2b), and the fit of the rate data to a hyperbola results in a maximum rate of 13.6 s^{-1} . The rate constant for dissociation is 13.6 s^{-1} and is significantly less than ATP hydrolysis at the active site occurring at 100 s^{-1} . This result therefore establishes that ATP hydrolysis occurs before K401 crossbridge dissociation and while K401 is still bound to the microtubule (Fig. 1, scheme 1). Furthermore, the rate constant for K401 dissociation is comparable to, and may therefore limit, the maximum steady-state turnover rate (see Discussion).

Attempts to measure the kinetics of K401 dissociation in the absence of added 100 mM KCl were unsuccessful. Subsequent analysis showed that kinesin was not completely released from the microtubule in a single turnover in the absence of added KCl. Experiments at low salt (Fig. 3b) show that kinesin goes through multiple turnovers of ATP per encounter with a microtubule before reaching steady state, and without significant release of kinesin from the microtubule (see later). To determine the effect of salt on ATP turnover, steady-state and rapid quench

[†] Present address: Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA.

[§] To whom correspondence should be addressed.

Scheme 1

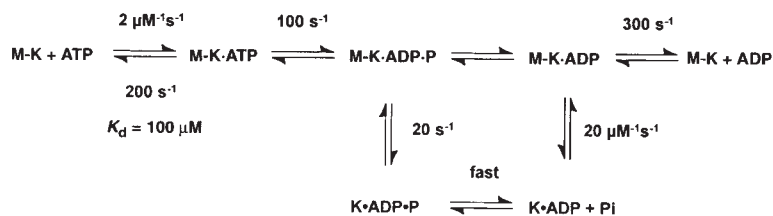
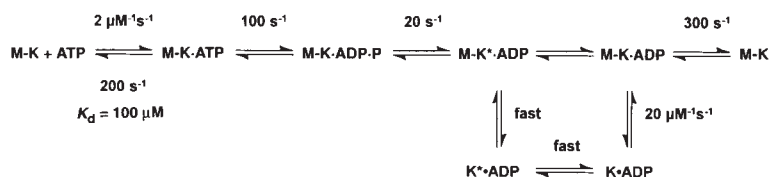


FIG. 1 Schemes 1 and 2 discussed in the text. Steady-state kinetic parameters are as follows: $k_{\text{cat}} = 20 \text{ s}^{-1}$; $K_{\text{m,ATP}} = 62 \mu\text{M}$; and $K_{0.5,\text{MT}} = 1 \mu\text{M}$.

Scheme 2



experiments were performed to examine the kinetics of ATP hydrolysis. Steady-state analysis showed that k_{cat} is unaffected by the added 100 mM KCl (20 s^{-1}) but that the K_{m} for microtubules is increased dramatically (data not shown). Figure 2c shows that the kinetics of ATP hydrolysis by the M-K401 complex during the first turnover (0–30 ms) were not affected by

the presence of the added KCl. The KCl, however, did affect subsequent turnovers of ATP, as shown by the linear phase of each transient in Fig. 2c, presumably by slowing microtubule rebinding and affecting the activation of product release. In the absence of added KCl, the rate of the linear phase is 22 s^{-1} , which is comparable to independent steady-state measurements

FIG. 2 Dissociation of the M-K complex. Mg-ATP was rapidly mixed with preformed M-K401 complexes and monitored in a stopped-flow instrument to measure the rate of kinesin dissociation from the microtubule. **a**, The stopped-flow record with $1 \mu\text{M}$ K401, $0.75 \mu\text{M}$ tubulin, 1 mM Mg-ATP, and 100 mM KCl (final concentrations). The solid line is the best fit of the data to a double exponential, with k_{obs} of the initial fast phase as $13.4 \pm 0.3 \text{ s}^{-1}$. The slow phase observed in the absence of ATP was ignored as it was too slow to be part of the ATPase cycle and was independent of ATP concentration. **b**, Rate of K401 dissociation from the M-K complex ($1 \mu\text{M}$ K401, $0.75 \mu\text{M}$ tubulin) as a function of Mg-ATP concentration. The solid line is the fit of the data to a hyperbola with $k_{\text{diss}} = 13.6 \pm 0.6 \text{ s}^{-1}$. **c**, Pre-steady-state kinetics of the M-K complex ($10 \mu\text{M}$ K401, $12 \mu\text{M}$ tubulin) at $400 \mu\text{M}$ [$\alpha\text{-}^{32}\text{P}$]Mg-ATP in the presence (■) and absence (●) of 100 mM KCl. Smooth lines show the global fit of the data by computer simulation to the mechanism in scheme 1 of Fig. 1, with $K_{\text{on,ATP}} = 0.9 \mu\text{M}^{-1} \text{ s}^{-1}$; $k_{\text{off,ATP}} = 200 \text{ s}^{-1}$ and $k_{\text{hydroly}} = 75 \text{ s}^{-1}$. In the absence of added KCl, $k_{\text{ss}} = 22 \text{ s}^{-1}$, and in the presence of 100 mM KCl, $k_{\text{ss}} = 11 \text{ s}^{-1}$. The rate constant, k_{ss} , represents the rate-limiting step in the pathway that limits subsequent steady-state turnovers of ATP. The linear phase of each transient represents subsequent turnover and corresponds to steady state. **METHODS**. K401 and tubulin were prepared as described⁹. Microtubules were assembled from soluble tubulin (cold depolymerized and clarified) and stabilized with $20 \mu\text{M}$ taxol. All assays were done at 25°C in ATPase buffer (20 mM HEPES, pH 7.2 with KOH, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate, 1 mM DTT), with concentrations being the final values after mixing. One syringe contained the preformed M-K401 complex (all M-K401 complexes stabilized with $10 \mu\text{M}$ taxol), and the other syringe contained Mg-ATP plus KCl. Stopped-flow experiments were performed using a KinTek StopFlow System (model SF-2001, KinTek). Turbidity was monitored at 340 nm ; each trace or data point represents the average of 4–8 stopped-flow traces. The data **a** were fitted to a double exponential to correct for a slow change seen in the signal. The rate of the slow phase did not increase with increasing ATP concentration and was too slow to be on the pathway. In a control experiment, the M-K401 complex was rapidly mixed with buffer in the presence of 100 mM KCl. There was no fast change in turbidity. The rapid quench experiment (**c**) was done as described¹¹ using a rapid-quench-flow instrument (model RQF-3, KinTek). The reaction was terminated by 2 M HCl, then mixed with chloroform to denature the protein and 2 M Tris– 3 M NaOH to neutralize the reaction mixture. The product [$\alpha\text{-}^{32}\text{P}$]ADP was separated from [$\alpha\text{-}^{32}\text{P}$]ATP by PEI-cellulose thin-layer chromatography (elution buffer, 0.6 M potassium phosphate, pH 3.4), and radiolabelled ADP and ATP were quantified using a Betascope 603 blot analyser (Betagen). Kinetics were modelled by numerical integration using the KINSIM program²².

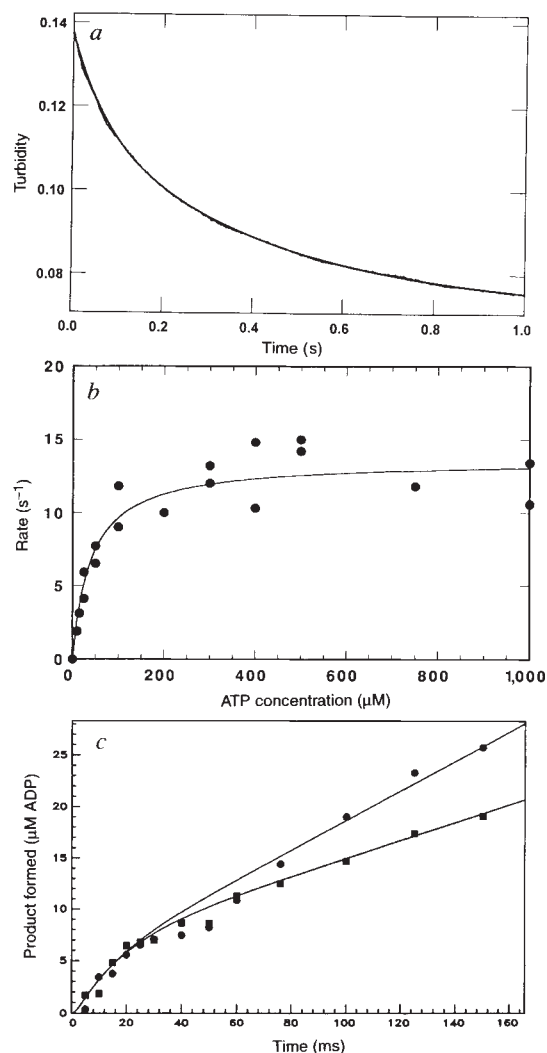
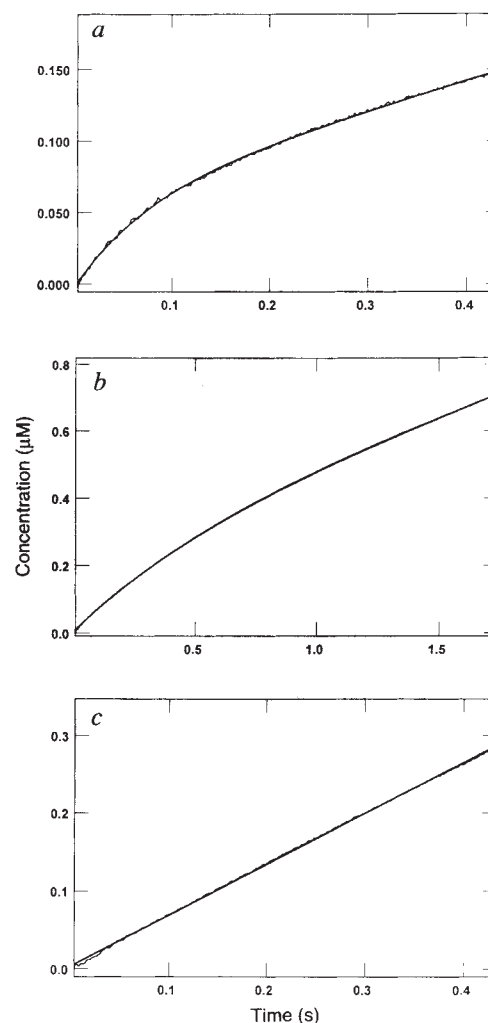


FIG. 3 Phosphate release kinetics. Real-time kinetics of P_i release were measured directly using a fluorescent coupled assay¹⁵. **a**, Stopped-flow trace for rapid mixing of M-K401 complex (0.05 μ M K401, 0.075 μ M tubulin) with 500 μ M Mg-ATP plus 100 mM KCl. Data are expressed as μ M P_i formed as a function of time (fluorescence signal provided 2 volts, equivalent to 1 μ M P_i). The smooth line shows the fit of the data to the burst equation ($y = A1 * \exp(-k_1 t) + k_2 t + C$) which gave the rate (k_1) of P_i release during the first turnover as $13.1 \pm 0.2 \text{ s}^{-1}$, the amplitude of the burst ($A1$) as 0.5 μ M, and the observed rate of P_i release during subsequent turnovers (k_2) as $4.2 \pm 0.02 \text{ s}^{-1}$. **b**, Stopped-flow trace as for **a** but without KCl. The M-K401 complex was formed at 0.05 μ M K401 and 0.075 μ M tubulin with 500 μ M Mg-ATP. Data were fitted to the burst equation to give an apparent burst rate of 1.8 s^{-1} and an amplitude of 0.25 μ M (corresponding to 5 ATP per site). **c**, Stopped-flow trace in the absence of added KCl but with a higher concentration of microtubules. The M-K401 complex was formed at 0.05 μ M K401 and 2 μ M tubulin and rapidly mixed with Mg-ATP to a final concentration of 500 μ M, with 50 mM potassium acetate. No burst of product formation was evident during the first turnover, and the data were fitted to a straight line with an observed rate of $13.4 \pm 0.04 \text{ s}^{-1}$.

METHODS. The coupled assay system¹⁵ contained the A197C mutant of the *E. coli* phosphate-binding protein (PBP) covalently modified at cysteine 197 by *N*-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide (MDCC) to produce the fluorescent reporter molecule, MDCC-PBP. The probe binds P_i rapidly ($1.36 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and tightly ($K_d \sim 0.1 \mu\text{M}$), affording a 5-fold change in fluorescence. Background phosphate was removed from the buffer and solutions by the 'P_i mop', consisting of 7-methylguanosine (0.5 mM) and purine nucleoside phosphorylase (0.5 units per ml). P_i release from the M-K401 complex upon mixing with Mg-ATP was determined by measurement of the fluorescence change that occurred with P_i binding by MDCC-PBP (2 μ M). The data, recorded as the relative fluorescence (volts), was converted to concentration (μ M) based on preliminary experiments in which the MDCC-PBP was rapidly mixed with excess KH_2PO_4 (data not shown). Stopped-flow experiments used a Hg arc lamp, with excitation at 425 nm; emission was observed using a 450 nm cutoff long-wave pass filter. The M-K401 complex was preformed in 2 μ M K401 and 3 μ M tubulin and diluted to 0.05 μ M K401, 0.075 μ M tubulin to ensure that K401 was in a dimer state.



for the M-K401 complex¹¹ ($k_{\text{cat}} = 20 \pm 2 \text{ s}^{-1}$ per site for this K401 preparation). The rate of the linear phase is 11 s^{-1} when 100 mM KCl is present with the Mg-ATP, and this rate is comparable to measurements made during steady-state turnover in the presence of 100 mM KCl and 12 μ M tubulin. The maximum rate was still $20 \pm 2 \text{ s}^{-1}$ at the higher salt concentration, but greater tubulin concentrations were required to achieve the maximal activation. Thus these results show that addition of 100 mM KCl with Mg-ATP does not significantly affect the rate constants governing the first turnover, namely ATP binding and hydrolysis, yet slows microtubule rebinding sufficiently to allow the kinetics of K401 dissociation from the microtubule to be measured.

Phosphate release kinetics

ADP remains tightly bound to kinesin in the absence of microtubules in both native and bacterially expressed kinesin^{9,13,14}. However, no direct measurements of inorganic phosphate (P_i) release under any conditions or ADP release in the presence of microtubules have been reported. Figure 3 shows the kinetics of P_i release for the M-K401 complex during the first turnover using a newly developed fluorescence-detected phosphate assay¹⁵. Figure 3a shows the time-dependence of the fluorescence change after mixing the M-K401 complex with Mg-ATP plus 100 mM KCl. The fluorescence change shows a burst of phosphate release (amplitude, 0.05 μ M; 1 ATP per site) and a rate of 13.1 s^{-1} , followed by a slower linear phase of P_i release during subsequent turnovers of ATP. The experiment was done using a very low concentration of microtubules in the presence of

100 mM KCl added with the ATP so that the rate of the subsequent turnovers (during the steady state) would be less than the rate of the first turnover. These conditions permitted observation of the initial burst of product formation during the first turnover. The results demonstrated that phosphate release occurs at a rate equal to the rate of dissociation of the microtubule-kinesin complex.

An identical experiment was performed in the absence of added KCl (Fig. 3b). The data fitted a burst equation with a rate of 1.8 s^{-1} and an amplitude of 0.25 μ M (5 ATP per site). So under these conditions, the burst of phosphate formation is a function of processive ATP hydrolysis, whereby several ATP molecules are hydrolysed per site before the steady-state distribution of kinesin on and off the microtubule is attained; that is, kinesin continues along the microtubule for several rounds of ATP hydrolysis before dissociating. The kinetics of the processive hydrolysis are a function of the relative rates of ATP-induced dissociation and rebinding of the kinesin intermediate state with one head released from the microtubule (see below and Fig. 6). The observed burst amplitude of 5 ATP per site should be corrected for the reduction in amplitude due to the fast steady-state rate relative to the slow observed burst rate. A simple burst model would lead to a corrected burst amplitude of 20 ATP per site. However, more rigorous analysis of the kinetics of this reaction by numerical integration shows that the time course can be fitted by a model with a rebinding rate of $\sim 200 \text{ s}^{-1}$, indicating a processivity of ten molecules of ATP hydrolysed per kinesin head before complete release from the microtubule (see below). This experiment provides a direct demonstration

and quantification of processive ATP hydrolysis by kinesin at low microtubule concentrations.

In a similar experiment using a higher concentration of microtubules (2 μM tubulin) and without added KCl (Fig. 3c), a burst is not seen because the high microtubule concentration leads to maximum activation of turnover, which is limited by the rate of phosphate release (and microtubule release). The rate of the linear phase at 2 μM tubulin (13 s^{-1}) is equal to that observed by steady-state measurements at this subsaturating microtubule concentration (data not shown).

Our kinetic data eliminate any model that assumes fast P_i release followed by slow dissociation of kinesin from the M-K complex. Rather, they indicate that the rate of P_i release is equal to the rate of K401 dissociation from the M-K complex. Thus, it is difficult to establish which reaction occurred first, and two possible pathways exist (Fig. 1, schemes 1 and 2). No lag was apparent in the kinetics of either K401 dissociation or P_i release, implying that the first reaction in the sequence occurs at 13 s^{-1} and is followed immediately by the second reaction at a much faster rate. The lower limit for the second step must be $\sim 100 \text{ s}^{-1}$; otherwise a lag in the kinetics of the second reaction would be detected. A more complete discussion of the kinesin dissociation and P_i release kinetics will be presented in the discussion.

K401 · ADP binding to the microtubule

Because native kinesin and K401 purify with tightly bound $\text{ADP}^{9,13,14}$ it has been assumed that the K · ADP intermediate rebinds to microtubules to complete the cycle (Fig. 1, schemes 1 and 2). The rate of binding of K401 · ADP was measured by stopped-flow turbidity measurements. Figure 4a shows a representative experiment at 5 μM tubulin, giving a rate of binding of 90 s^{-1} . The rate of binding is dependent on microtubule concentration (Fig. 4b), increasing linearly with microtubule concentration. The rate defines the apparent second-order rate constant for K · ADP binding to the microtubule as 20 $\mu\text{M}^{-1} \text{ s}^{-1}$ (Fig. 1, scheme 1). This rate constant for microtubule binding is

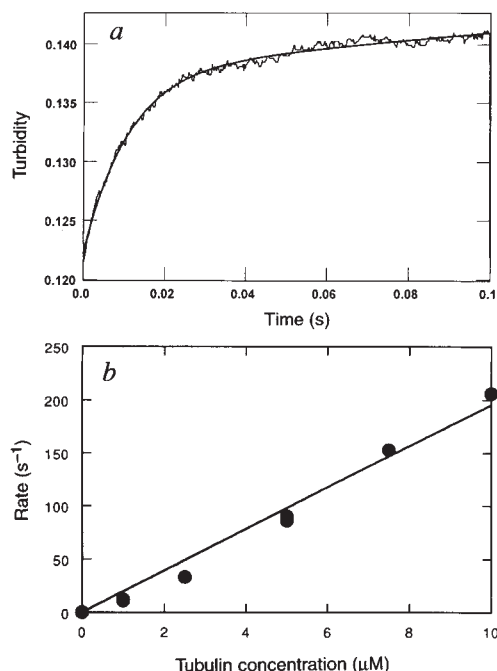


FIG. 4 Kinetics of K · ADP binding to the microtubule. *a*, Stopped-flow record for an experiment in which 2 μM K401 · ADP was rapidly mixed with 5 μM tubulin. Smooth line shows the fit to an exponential followed by a linear phase with the observed exponential rate at 90 s^{-1} . *b*, Microtubule concentration dependence of the rate of binding by K401. Data were fitted to a straight line; the slope gives the apparent second-order rate constant for microtubule binding ($19.5 \pm 0.7 \mu\text{M}^{-1} \text{ s}^{-1}$).

equal to the apparent second-order rate constant for microtubule activation of steady-state turnover (Fig. 3c) obtained from the initial slope of the microtubule-concentration dependence of the ATPase rate (equal to the ratio $k_{\text{cat}}/K_{0.5, \text{MT}} = 20 \mu\text{M}^{-1} \text{ s}^{-1}$). It is therefore reasonable to suggest that both assays are measuring the same rate-limiting step at low microtubule concentrations; namely, the binding of the K · ADP intermediate to microtubules.

ADP release from the M-K · ADP intermediate

To complete the cycle, the rate of ADP release from the M-K · ADP intermediate was measured using the fluorescent ADP analogue 2'(3')-*O*-(*N*-methylanthraniloyl)-adenosine 5'-triphosphate (mantADP), previously used in kinetic measurements for both skeletal myosin¹⁶ and native kinesin¹⁴. K401 was labelled with mantADP by exchanging the ADP at the active site with mantADP. The K401-mantADP complex was then rapidly mixed with microtubules in the presence or absence of ATP in the stopped-flow, and the release of mantADP from kinesin was monitored as a change in fluorescence. Figure 5a shows the time-dependence of the fluorescence change, fitted to a single exponential. The rate increased with microtubule concentration (Fig. 5b) and the fit of the data to a hyperbola yielded a maximum rate of 306 s^{-1} (Fig. 1, scheme 1) and a second-order binding rate constant of 20 $\mu\text{M}^{-1} \text{ s}^{-1}$. Note that Woodward *et al.*¹⁶ determined the rate of mantADP product release for actomyosin as 393 s^{-1} .

Several observations lead to the conclusion that the stopped-flow signal is a function of the release of mantADP from the M-K-mantADP intermediate, rather than the binding of microtubules to K-mantADP. First, the rate saturates at increasing microtubule concentration. Second, similar results were obtained in the presence and absence of a high concentration of Mg-ATP, which was included with the microtubules to ensure that mantADP was released.

The experiment presented in Fig. 5c compares Mg-mantATP with Mg-ATP as a substrate for the M-K complex. The steady-state parameters for ATP were $k_{\text{cat}} = 19 \text{ s}^{-1}$ and $K_{\text{m,ATP}} = 62 \mu\text{M}$; and $k_{\text{cat}} = 19 \text{ s}^{-1}$ and $K_{\text{m,mantATP}} = 151 \mu\text{M}$ for mantATP. The steady-state k_{cat} and K_{m} parameters are a function of the rates of substrate binding and the rate-limiting step of the reaction. The similar k_{cat} values for ATP and mantATP suggest that the rapid rate of mantADP release, which is faster than steady-state turnover, also holds for ADP product release.

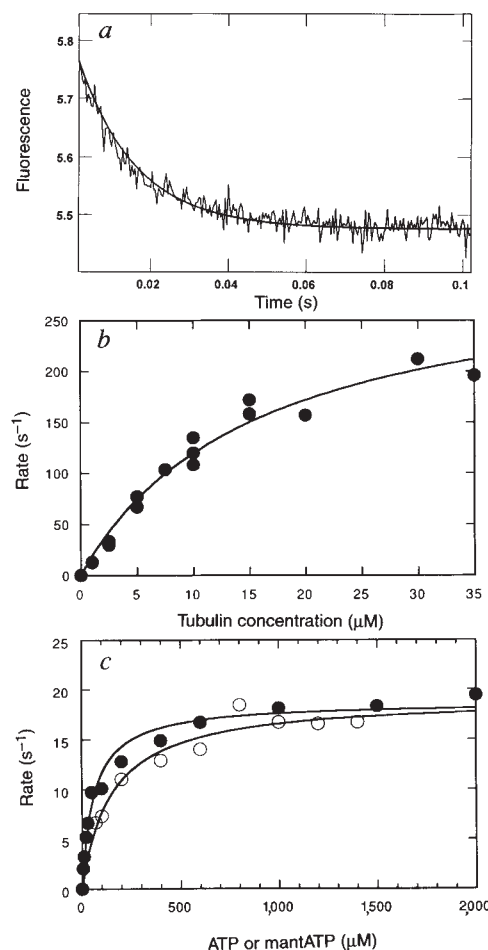
Sequential release of kinesin heads

The rate constant determined for the rate-limiting step in the pathway at 13 s^{-1} (Figs 2, 3) presented a puzzle. Steady-state experiments yielded a k_{cat} of $20 \pm 2 \text{ s}^{-1}$ for this preparation of K401, which is significantly greater than 13 s^{-1} . There appeared to be an inconsistency in pre-steady-state versus steady-state kinetics in that no step in the pathway can have a rate significantly less than steady-state turnover. To resolve this, we evaluated a model invoking sequential head release from the microtubule using computer simulation (Fig. 6). The net reaction involving the sequential release of two heads, each at 20 s^{-1} , should in a double exponential trace which can be fitted adequately to a single exponential to give an apparent rate of 13 s^{-1} . The model still predicts a steady-state turnover rate of 20 s^{-1} per site. This analysis therefore accounts for the slower rates measured for K401 dissociation or P_i release and reconciles these results with the observed steady-state turnover. The data can be fitted using other combinations of rate constants, as long as the requirement of an average rate of 20 s^{-1} per head is achieved and the single turnover rate of 13 s^{-1} can be predicted with no lag in the dissociation kinetics, although these requirements restrict the choice of rate constants considerably.

We conclude that interaction of the two heads with each other on the surface of a microtubule changes their ATPase kinetics so that their dissociation occurs sequentially. The kinetic data

FIG. 5 Kinetics of mantADP release from the M-K · mantADP complex. **a**, Representative stopped-flow record in which 2 μM K401 · mantADP was rapidly mixed with microtubules (5 μM tubulin) plus 1 mM Mg-ATP. Solid line is the best fit of the data to a single exponential with $k_{\text{obs}} = 67 \text{ s}^{-1}$. **b**, The microtubule concentration dependence of the rate of mantADP release from the M-K · mantADP complex (2 μM K401 · mantADP). The fit of the data to a hyperbola gives the maximum rate of $306 \pm 25 \text{ s}^{-1}$. **c**, Steady-state ATPase (●) and mantATPase (○) of the M-K401 complex with 0.2 μM K401 and microtubules at 12 μM tubulin. Each data set was fitted to a hyperbola to determine the steady-state parameters for each substrate: for Mg-ATP $k_{\text{cat}} = 18.6 \pm 0.6 \text{ s}^{-1}$ and $K_{\text{m,ATP}} = 61.5 \pm 8.4 \mu\text{M}$; for Mg-mantATP, $k_{\text{cat}} = 19.0 \pm 0.9 \text{ s}^{-1}$ and $K_{\text{m,mantATP}} = 151 \pm 30 \mu\text{M}$.

METHODS. K · mantADP was prepared by incubating K401 as purified (ADP tightly bound at the active site) with mantADP (Molecular Probes) at 25 °C with 40 μM K401 plus 40 μM Mg-mantADP for 20 min before preparing the M-K · mantADP complex (for some experiments, mantADP was in excess of K401). There was no difference in signal or observed rates with labelling 1:1 (K401:mantADP) or with mantADP in excess (labelling at 1:4). The purity of mantADP and mantATP was confirmed by thin-layer chromatography (silica gel 60, F_{254} in 1-propanol/ NH_4OH /water 6:3:1, v/v, containing 0.5 g l^{-1} EDTA); *N*-methylanthraniloyl derivatives had the absorption and fluorescence spectra previously described²³. The steady state for ATPase in **c** was determined as described⁹ using Mg- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. For mantATP experiments, P_i was quantified by malachite green assay²⁴.



rule out a model in which there is random release of either of two identical heads bound to the microtubule; a model invoking random and independent heads would predict a net reaction occurring during a single turnover at 20 s^{-1} , rather than at 13 s^{-1} as we observed. Accordingly, the two heads must interact while bound to the microtubule to induce an asymmetry leading to dissociation of only one head at a time.

Discussion

The mechanism shown in Fig. 1, scheme 1 accounts quantitatively for the steady-state kinetic parameters as well as the elementary rate constants of the steps measured directly for each partial reaction in the pathway. Rapid chemical quench-flow experiments previously defined the kinetics of ATP binding and ATP hydrolysis¹¹. These results show that ATP binds rapidly but weakly (apparent $K_{\text{d,ATP}} = 100 \mu\text{M}$) to the M-K complex and is followed by fast ATP hydrolysis. Dissociation of K401 from the microtubule is a slow reaction (20 s^{-1}) relative to the rate of ATP hydrolysis (100 s^{-1}), showing that ATP hydrolysis occurs with kinesin still bound to the microtubule. Furthermore, dissociation of kinesin from the microtubule and P_i release occur at the same rate (Figs 2, 3), with no lag in the kinetics of either reaction. These results indicate that in the two-step sequence, the first step is slow and rate-limiting and the second step is much faster. Although either of the schemes shown in Fig. 1 could account for the results, we favour scheme 1. Kinesin dissociation from the microtubule is the slow and rate-limiting step and is followed by rapid P_i release from the K · ADP · P_i intermediate. The cycle is then completed by the rapid rebinding of the K · ADP intermediate to the microtubule, followed by fast ADP product release from the M · K · ADP intermediate.

The alternative pathway shown in scheme 2 of Fig. 1 is also consistent with our data. According to this model, phosphate release occurs first and is the rate-limiting step, and is followed by rapid dissociation of $\text{K}^* \cdot \text{ADP}$ from the microtubule. This model is attractive because it allows for an additional step leading to phosphate release as a potential site for the power stroke while the kinesin is still attached to the microtubule. But to account for an obligatory dissociation from the microtubule during the pathway, scheme 2 requires that there be a new $\text{K}^* \cdot \text{ADP}$ state. Because there is no direct evidence for this species, scheme 1 represents the simpler model to account for the available data.

The pathway accounts for the features of kinesin-promoted motility found *in vitro* single-motor assays^{6,17,18}. In these assays, a single kinesin molecule (a dimer with two motor domains) translocates over long distances along a single microtubule at maximal rates and without dissociating, making kinesin a highly processive motor compared with myosin, as single molecules of skeletal myosin will not promote directed actin-based movements⁷. Moreover, the rate of movement for a kinesin construct of similar length¹⁹ (K410 at 400 nm s^{-1}) corresponds to the step size of 8 nm and a turnover rate of 25 ATP per second per site; our ATPase rates can thus account for the motility.

The kinetic results presented in Figs 3 and 6 indicate that the processivity of kinesin is accomplished in part by coordination of ATPase cycles of the two heads of the kinesin dimer. In this model for motility, the first head dissociates from the microtubule before the second; once the first head is dissociated, it can rapidly rebound to the microtubule at the next site on the microtubule lattice, establishing processive ATP hydrolysis. Therefore dimeric kinesin can run the length of the microtubule with multiple ATPs hydrolysed per site, without kinesin release and diffusion from the microtubule.

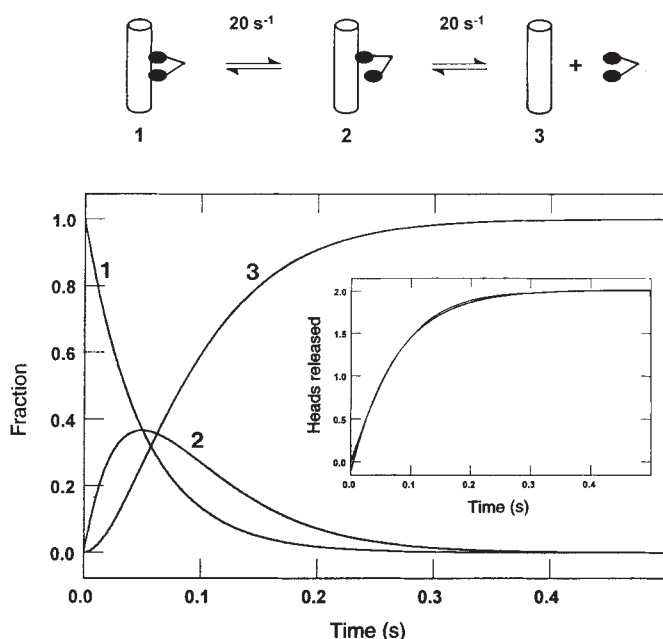


FIG. 6 Sequential release of the kinesin heads from the microtubule. Computer modelling (KINSIM) of the kinetic data predicted the time courses shown as dimeric kinesin progresses through a complete cycle (2 ATPs hydrolysed). Curve 1 represents the species with both kinesin heads bound to the microtubule. Curve 2 shows the appearance of the intermediate that results after the first head is released, and curve 3 shows the appearance of the intermediate in which both heads are dissociated from the microtubule. Inset: simulated kinetics of the sequential release of two heads, with each species contributing equally to the signal and each step occurring at 20 s^{-1} . Superimposed on this trace is the best fit to a single exponential giving a rate of 13.3 s^{-1} .

A second mechanism that ensures that the kinesin dimer continues its translocation along the microtubule is the very fast rebinding of the completely dissociated kinesin intermediate ($\text{K} \cdot \text{ADP}$) to the microtubule. The rate of rebinding is determined by the local microtubule concentration and if this is estimated as $\sim 1 \text{ mM}$ near a kinesin head after its release (a tubulin dimer contained in a $10 \times 10 \times 10 \text{ nm}$ cube), kinesin could rebound to the microtubule at a rate of $20,000 \text{ s}^{-1}$, giving a lifetime for the detached state of $\sim 50 \mu\text{s}$. The processivity of kinesin in motility assays is therefore accomplished in part by a brief time in the detached state and the sequential release of the kinesin heads, and is further enhanced by the fast rebinding rates and the hindered diffusion of kinesin while bound to a large bead.

The processive ATP hydrolysis by kinesin can be defined as the number of ATP molecules hydrolysed per ATPase site for each encounter of kinesin with a microtubule. We have shown that the processivity is ~ 10 ATPs hydrolysed per site (Fig. 3b) as a result of the intermediate state rebinding at a rate of 200 s^{-1} (species 2 in Fig. 6) to the microtubule. Although our model and

that of Hackney²⁰ both describe a hand-over-hand mechanism of force production, further experiments will be required to distinguish between them.

The mechanism of action of kinesin shown in schemes 1 and 2 of Fig. 1 clarifies the behaviour of the non-hydrolysable ATP analogue AMP-PNP. As AMP-PNP promotes binding of kinesin with microtubules²¹, it was thought that ATP binding might stimulate formation of an $\text{M-K} \cdot \text{ATP}$ complex by an opposite mechanism to that of the actomyosin and microtubule-dynein systems. However, the microtubule-kinesin complex can be formed in the absence of AMP-PNP^{9,10}. Our results suggest that AMP-PNP may simply lock the kinesin in a bound state in view of the fact that ATP hydrolysis is required for kinesin crossbridge release from the microtubule.

As we do not yet have all the information to calculate the changes in free energy at each step in the cycle, we cannot identify the step in the pathway for force production, although the power stroke will probably occur during the longest attached state. Because the only slow step limits kinesin release after ATP hydrolysis, a conformational change associated with the 20 s^{-1} rate could be the point in the cycle at which the power stroke takes place. In scheme 1 (Fig. 1), the 20 s^{-1} step could be a function of kinesin isomerization occurring after ATP hydrolysis and before its dissociation from the microtubule; once the power stroke is complete at 20 s^{-1} , the conformational change would cause the rapid release of the $\text{K} \cdot \text{ADP} \cdot \text{P}_i$ intermediate from the microtubule.

Alternatively, in scheme 2 of Fig. 1, the power stroke could occur as a slow conformational change of the $\text{M-K} \cdot \text{ADP} \cdot \text{P}_i$ intermediate at 20 s^{-1} ; once it is complete, P_i release and kinesin dissociation would be rapid. In both schemes, the power stroke would occur as a slow conformational change at 20 s^{-1} , followed by rapid release of the kinesin crossbridge from the microtubule. Other possible stages in the cycle for force generation include an isomerization after ATP binding and before hydrolysis, or a conformational change of the $\text{M-K} \cdot \text{ADP}$ intermediate before ADP product release, but are unlikely because of the short lifetimes of these intermediates.

The microtubule-kinesin pathway differs from those of actomyosin and dynein in ways that are significant for the biology of these motor systems. Both myosin and axonemal dynein operate in macromolecular assemblies and the lifetime of the attached state is quite short in both systems (0.5 ms). Under conditions of maximal actin activation in solution, most of the myosin is dissociated, consistent with concerted force production by multiple motors in an ordered array. In comparison to actomyosin, kinesin dissociates from its filament 100-fold slower and rebinds 2,000-fold faster. Kinesin is a cytoplasmic motor operating as a single motor (or with a few motors) on an organelle, and it must translocate an organelle over long distances down the axon along a microtubule. Kinesin keeps the organelle in contact with the microtubule by the sequential release of individual heads occurring late in the ATPase cycle, followed by the rapid rebinding to the microtubule. This mechanism coordinates the ATPase cycles of the two heads of dimeric kinesin and establishes the processivity observed as the tendency of kinesin to run for long distances along the microtubule. □

Received 5 October 1994; accepted 17 January 1995.

- Vale, R. D., Reese, T. S. & Sheetz, M. P. *Cell* **42**, 39–50 (1985).
- Brady, S. T. *Nature* **317**, 73–75 (1985).
- Scholey, J. M., Porter, M. E., Grissom, P. M. & McIntosh, J. R. *Nature* **318**, 483–486 (1985).
- Taylor, E. W. *The Heart and Cardiovascular System* 2nd edn 1281–1293 (Raven, New York, 1992).
- Johnson, K. A. *Rev. Biophys. biophys. Chem.* **14**, 161–188 (1985).
- Howard, J., Hudspeth, A. J. & Vale, R. D. *Nature* **342**, 154–158 (1989).
- Uyeda, R. Q. P., Warrick, H. M., Kron, S. J. & Spudis, J. A. *Nature* **352**, 307–311 (1991).
- Greene, L. E. & Eisenberg, E. *J. biol. Chem.* **255**, 543–548 (1980).
- Gilbert, S. P. & Johnson, K. A. *Biochemistry* **32**, 4677–4684 (1993).
- Harrison, B. C. et al. *Nature* **362**, 73–75 (1993).
- Gilbert, S. P. & Johnson, K. A. *Biochemistry* **33**, 1951–1960 (1994).
- Holzbaur, E. L. F. & Johnson, K. A. *Biochemistry* **28**, 7010–7016 (1989).
- Hackney, D. D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6314–6318 (1988).

- Sadhu, A. & Taylor, E. W. *J. biol. Chem.* **267**, 11352–11359 (1992).
- Brune, M., Hunter, J. L., Corrie, J. E. T. & Webb, M. R. *Biochemistry* **33**, 8262–8271 (1994).
- Woodward, S. K. A., Eccleston, J. F. & Geeves, M. A. *Biochemistry* **30**, 422–430 (1991).
- Block, S. M., Goldstein, L. S. B. & Schnapp, B. J. *Nature* **348**, 348–352 (1990).
- Romberg, L. & Vale, R. D. *Nature* **361**, 168–170 (1993).
- Stewart, R. J., Thaler, J. P. & Goldstein, L. S. B. *Proc. natn. Acad. Sci. U.S.A.* **90**, 5209–5213 (1993).
- Hackney, D. D. *Proc. natn. Acad. Sci. U.S.A.* **91**, 6865–6869 (1994).
- Lasek, R. J. & Brady, S. T. *Nature* **316**, 645–647 (1985).
- Barshop, B. A., Wrenn, R. F. & Frieden, C. *Analyt. Biochem.* **130**, 134–145 (1983).
- Hiratsuka, T. *Biochim. biophys. Acta* **742**, 496–508 (1983).
- Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. *Analyt. Biochem.* **100**, 95–97 (1979).

ACKNOWLEDGEMENTS. We thank N. R. Lomax of the National Cancer Institute for taxol. Supported by the NIH (K.A.J.) and a European Community twinning grant, the Human Frontiers Science Program and the MRC, UK (M.R.W.).

Detection of an oxygen atmosphere on Jupiter's moon Europa

D. T. Hall*, D. F. Strobel*, P. D. Feldman*,
M. A. McGrath† & H. A. Weaver†

* Center for Astrophysical Sciences, The Johns Hopkins University,
34th and Charles Streets, Baltimore, Maryland 21218, USA

† Space Telescope Science Institute, 3700 San Martin Drive,
Baltimore, Maryland 21218, USA

EUROPA, the second large satellite out from Jupiter, is roughly the size of Earth's Moon, but unlike the Moon, it has water ice on its surface¹. There have been suggestions that an oxygen atmosphere should accumulate around such a body, through reactions which break up the water molecules and form molecular hydrogen and oxygen^{2,3}. The lighter H₂ molecules would escape from Europa relatively easily, leaving behind an atmosphere rich in oxygen. Here we report the detection of atomic oxygen emission from Europa, which we interpret as being produced by the simultaneous dissociation and excitation of atmospheric O₂ by electrons from Jupiter's magnetosphere. Europa's molecular oxygen atmosphere is very tenuous, with a surface pressure about 10⁻¹¹ that of the Earth's atmosphere at sea level.

In an observation specifically designed to detect an oxygen atmosphere, the Hubble Space Telescope (HST) observed Europa during six consecutive spacecraft orbits on 2 June 1994 using the square 1.74 × 1.74 arcsec aperture and far-ultraviolet grating (G140L) of the Goddard High Resolution Spectrograph (GHRS). To minimize contamination by terrestrial oxygen day-glow, exposures were conducted when HST was in Earth's shadow. At a distance of 4.57 AU, the trailing hemisphere of Europa's solid surface (diameter 3,138 km) subtended an angle of 0.947 arcsec. During the first HST orbit, the telescope was aligned so that Europa was centred in the aperture; however, during the third orbital exposure, the telescope boresight drifted ~0.8 arcsec from this initial orientation. This drift means that much of Europa's disk may have been excluded from the aperture, possibly artificially depressing detected emission intensities.

Figure 1 shows the combined spectra in the 129–137 nm region. Three emission features are detected, with the characteristic instrumental spectral profile expected for a disk the diameter of Europa. Table 1 summarizes measured brightnesses. The weakest feature near 133.5 nm is interpreted as surface-reflected sunlight for two reasons. First, the solar C II 133.5-nm emission lines are the strongest in the 129–137 nm bandpass. Second, the measured spectral line profile is well matched by that calculated for a uniform Europa disk reflecting the solar 133.5-nm feature convolved with the GHRS line-spread function, which is a gaussian with full-width at half-maximum comparable to the spectral width of one detector diode (0.0572 nm). Using a solar 133.5-nm flux of $F = 0.7 \times 10^9$ photons cm⁻² s⁻¹ as measured at 1 AU during 1985 by the solar ultraviolet spectral irradiance monitor (SUSIM) experiment⁴, we estimate a geometric surface albedo of $2.3 \pm 0.8\%$ for the trailing hemisphere, significantly lower than the 5.5% reflectance of pure H₂O frost measured in the laboratory⁵. Although the measured albedo may have been artificially depressed by HST boresight pointing variations, the low value suggests the presence of an ultraviolet-darkening contaminant in Europa's icy surface material; this is consistent with observations at other wavelengths^{6–8}. To estimate the reflected-sunlight components of the two O I features we assume that the surface reflectance does not vary significantly in the 130–136 nm spectral range, and calculate the brightness using the measured albedo. SUSIM solar fluxes at 130.4 and 135.6 nm are $F = 8.4 \times 10^9$ and 2.6×10^9 photons cm⁻² s⁻¹ respectively⁴, and imply the reflected brightnesses given in Table 1.

The two O I emission features in the Europa spectrum are not of terrestrial origin because they have the spectral signature of Europa-disk emission. Diffuse monochromatic sources that fill the 1.74×1.74 arcsec aperture produce characteristically flat-topped instrumental line profiles spanning ~0.45 nm, as is observed for the Earth airglow hydrogen Lyman- α (121.6 nm) line. In contrast, the expected profile for uniform monochromatic Europa disk emission is a relatively sharply peaked function ~0.25 nm wide. We estimate an upper limit on the size of Europa's atmospheric emission region by comparing the detected shape of the O I 135.6-nm doublet with that predicted for a uniform disk source convolved with the GHRS line-spread function. The 135.6-nm feature is best fitted by an emitting disk 1.12 arcsec in diameter, ~20% larger than Europa's solid surface disk, but the data are also consistent with an emitting disk equal to the solid surface. The 2 σ upper limit for the emitting disk diameter is 1.5 arcsec, implying that the emissions originate from altitudes within ~900 km of Europa's solid surface, and possibly much closer.

To determine the abundance of oxygen in Europa's atmosphere we use extensive observations of Earth's oxygen airglow⁹ as a guideline, and model the emissions considering an atmosphere containing both O and O₂ as possible constituents. Our analysis indicates that, even though the observed emissions are emitted from atomic oxygen, such atoms comprise at most a small fraction of Europa's atmosphere, and the dominant form of oxygen is molecular rather than atomic. The relative intensities of the two O I spectral features are consistent with electron-impact dissociation of O₂ to produce excited oxygen atoms. Other excitation mechanisms (including resonance scattering by oxygen atoms) are negligible in comparison, and Europa's atmospheric layer of O₂ is too thin to absorb more than a few per cent of incident solar photons in the observed wavelength range. To demonstrate this, we consider the three most probable processes that can produce the observed emissions. The first two are electron-impact excitation of oxygen atoms,



and electron-impact dissociative excitation of O₂,

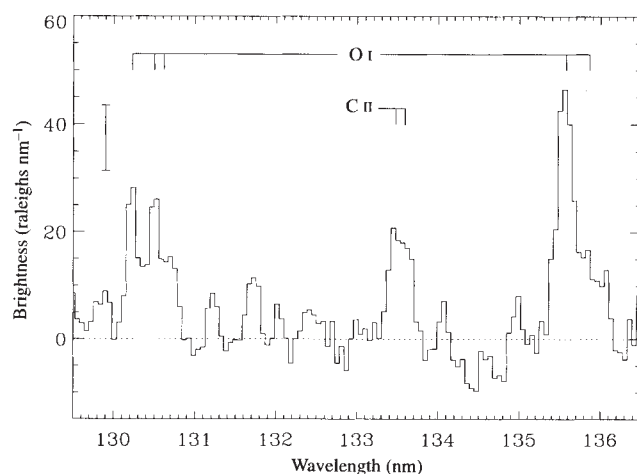


FIG. 1 Hubble Space Telescope spectrum of Europa (total exposure time, 7,290 s). The C II 133.5-nm feature is interpreted as reflected sunlight, whereas the O I 130.4- and 135.6-nm features are interpreted as being emitted mostly from a tenuous O₂ atmosphere enshrouding the icy satellite. The histogram shows the detected brightness in bins one GHRS detector-diode wide, assuming emission from a uniform Europa disk 0.947 arcsec in diameter. Instrumental substepping and combination/addition were performed to minimize the effects of diode-to-diode sensitivity variations. The spectrum has been smoothed over three bins, and a representative $\pm 1\sigma$ error bar is shown on the left.

TABLE 1 Observed Europa emission features

	Transition	Emission wavelength* (nm)	Measured brightness† (rayleighs)	Solar reflection (rayleighs)	Atmospheric emission (rayleighs)
O I	$2s^2 2p^4 \ ^3P \leftarrow 2s^2 2p^3 3s \ ^3S^o$	130.42–130.60	63 ± 11	26 ± 10	37 ± 15
C II	$2s^2 2p \ ^2P^o \leftarrow 2s^2 2p^2 \ ^2D$	133.45–133.57	30 ± 11	30 ± 11	—
O I	$2s^2 2p^4 \ ^3P \leftarrow 2s^2 2p^3 3s \ ^5S^o$	135.56–135.85	77 ± 13	8 ± 3	69 ± 13

* Wavelength range spanned by entire multiplet.

† Integrated brightness of observed feature, assuming uniform emission from Europa's disk, 0.947 arcsec in diameter. Quoted errors are $\pm 1\sigma$ statistical uncertainties.

where O* indicates an excited oxygen atom that subsequently emits a photon. The third process is resonance scattering of solar O I 130.4-nm photons from oxygen atoms. Preliminary analysis indicates that solar fluxes are insufficient for other photoexcitation processes to compete with these three. In addition, we tentatively dismiss other processes that might produce the emission, such as charged-particle impact excitation of solid substrates or other gaseous molecules (such as H₂O, O₃ or OH), or radiative recombination of singly ionized oxygen, but note that these processes should be investigated more completely.

Because Europa orbits within Jupiter's magnetosphere and resides in the outer regions of the plasma torus that is centred on the orbit of the volcanically active satellite Io, its atmosphere is penetrated by a significant flux of magnetospheric electrons. Near Europa's orbit the Voyager 1 spacecraft plasma science experiment detected a variable, non-maxwellian electron distribution with total density 30–50 cm⁻³, that may be attributed to cold and hot components^{10,11}. The dominant cold component had temperature $T_e = 15$ –25 eV, whereas the hot component, comprising 2–10% of the total density, had $T_e \geq 200$ eV. We calculate emission rates assuming representative electron parameters as follows: a total density $n_e = 40$ cm⁻³, a dominant cold component with $T_e = 20$ eV, and a 250-eV hot component with a 5% mixing ratio. In addition, we assume that the total oxygen gas column density is small enough that the electrons do not undergo multiple collisions as they penetrate Europa's atmosphere, which is strictly valid for column densities $\lesssim 10^{15}$ cm⁻². Our derived column density is somewhat larger than this, and therefore this analysis should be regarded as an estimate, to be superseded by a more rigorous calculation. Given the stated assumptions, the brightness in rayleighs (1 rayleigh is a unit of

surface brightness equivalent to an intensity of one million photons per square centimetre per second per 4π steradians) for a particular multiplet due to reaction (1) is

$$B_1 = \frac{n_e N(O) C_1}{10^6} \quad (3)$$

where $N(O)$ is the disk-averaged oxygen-atom column density (cm⁻²) and C_1 is the electron-impact rate coefficient (cm³ s⁻¹). A similar relation holds for reaction (2). Emission rate coefficients used in this analysis are $C_1(130.4 \text{ nm}) = 5.2 \times 10^{-9}$, $C_1(135.6 \text{ nm}) = 5.7 \times 10^{-10}$, $C_2(130.4 \text{ nm}) = 5.9 \times 10^{-10}$ and $C_2(135.6 \text{ nm}) = 1.1 \times 10^{-9}$ cm³ s⁻¹, and were calculated by integrating laboratory-measured cross-sections^{12–14} over the Io torus electron-velocity distribution function. Rates for reaction (1) also include contributions from cascading transitions, calculated using a multistate model of atomic oxygen¹⁵. Reaction (2) yields the emission ratio $C_2(135.6 \text{ nm})/C_2(130.4 \text{ nm}) = 1.9$, within measurement uncertainty of the observed ratio, whereas reaction (1) yields a distinctly different value of ~ 0.1 .

The O I 130.4-nm resonance scattering brightness may be written

$$B_3 = \frac{g N(O)}{10^6} \left[\frac{\varpi(\tau)}{\tau} \right] \quad (4)$$

where g is the resonance scattering probability (s⁻¹), τ is the line-centre optical depth, and $\varpi(\tau)$ is the effective resonant scattering albedo¹⁶, a function of optical depth. Using the SUSIM 130.4-nm multiplet solar flux of 8.4×10^9 photons cm⁻² s⁻¹ (at 1 AU) with its known solar line shape¹⁷, and accounting for Europa's average heliocentric radial speed of +10 km s⁻¹ during the observations, we calculate a scattering probability of $g = 4 \times 10^{-7}$ s⁻¹. To approximate the effects of multiple scattering, we use an effective optical depth $\tau = \sigma_O N(O)$, where σ_O is the line-centre cross-section for one of the individual lines of the O I 130.4-nm multiplet, and use values for $\varpi(\tau)$ calculated for individual resonance line scattering from a plane-parallel medium¹⁶. Preliminary analysis using a more detailed radiative transfer model designed specifically for O I 130.4 nm (ref. 18) indicates that this approximation is accurate to 15–20% for column densities $< 2 \times 10^{14}$ cm⁻².

To evaluate abundances of atomic and molecular oxygen, we model the atmospheric emissions by summing contributions from the three processes, and calculate a χ^2 goodness-of-fit indicator as a function of $N(O)$ and $N(O_2)$, as shown in Fig. 2. The analysis depends on the oxygen atom temperature (through the O I 130.4-nm resonance scattering cross-section) and the solid and dashed lines show confidence regions for gas temperatures of 1,000 K and 200 K respectively, which we consider reasonable bounds. Europa's atmosphere is dominated by O₂ rather than O, with $N(O_2) = (1.5 \pm 0.5) \times 10^{15}$ cm⁻², and most of the emission is produced by reaction (2). The observations are consistent with no atomic oxygen, and the 2.5σ upper limit is $N(O) < 2 \times 10^{14}$ cm⁻².

The detection of an oxygen atmosphere on Europa is significant because only three other planetary satellites (Io, Titan and Triton) are known to have atmospheres. The measured O₂ column density corresponds to a surface pressure of $\sim 10^{-11}$ bar, which agrees remarkably well with the predicted low-pressure

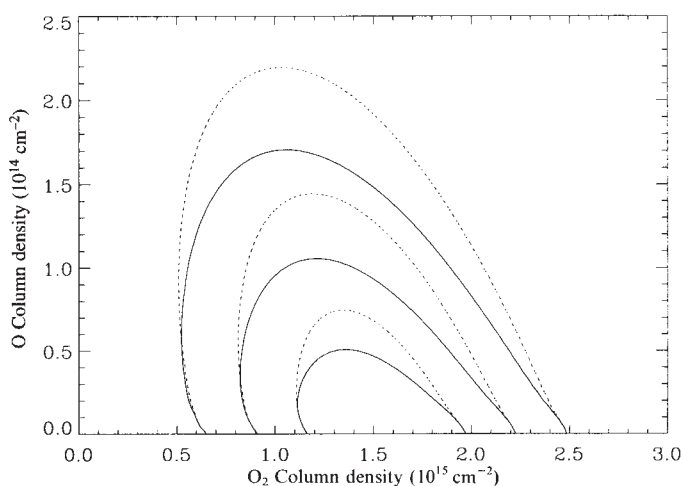


FIG. 2 Atomic- and molecular-oxygen disk-averaged column densities for Europa's atmosphere. Contours of the χ^2 goodness-of-fit indicator are shown for atomic-oxygen gas temperatures of 200 K (dashed lines) and 1,000 K (solid lines). Three contours for each temperature enclose 68.2, 95.4 and 99.7% confidence regions (conventional 1σ , 2σ and 3σ boundaries). The best-fit O₂ column density is 1.5×10^{15} cm⁻². The observations are consistent with zero atomic oxygen abundance, and the 2.5σ upper limit for the atomic-oxygen column density is a factor of 7.5 smaller than the best-fit O₂ column density.

equilibrium state of an evolved atmosphere on an icy satellite³. Candidate gas production processes on Europa's icy surface include H₂O sublimation, charged-particle sputtering and micrometeorite impacts. Of these, sputtering probably dominates the effects of both sublimation and micrometeorites because Europa's surface is subject to a significant flux of charged particles^{19,20}. Ironically, even though magnetospheric charged particles may provide the dominant source for the atmospheric gas, they should also tend to erode the atmosphere, competing with thermal and other escape processes. □

Received 9 November 1994; accepted 6 January 1995.

1. Malin, M. C. & Pieri, D. C. in *Satellites* (eds Burns, J. A. & Matthews, M. S.) 689–717 (Univ. Arizona Press, Tucson, 1986).
2. Yung, Y. L. & McElroy, M. B. *Icarus* **20**, 97–103 (1977).
3. Kumar, S. & Hunten, D. M. in *Satellites of Jupiter* (ed. Morrison, D.) 782–806 (Univ. Arizona Press, Tucson, 1982).
4. Vanhoosier, M. E., Bartoe, J. D. F., Brueckner, G. E. & Prinz, D. K. *Astro. Lett. Commun.* **27**, 163–168 (1988).
5. Hapke, B., Wells, E. & Wagner, J. *Icarus* **47**, 361–367 (1981).
6. Nelson, R. M. et al. *Icarus* **72**, 358–380 (1987).
7. Lane, A. L., Nelson, R. M. & Matson, D. L. *Nature* **292**, 38–39 (1981).
8. Clark, R. N. *Icarus* **44**, 388–409 (1980).
9. Meier, R. R. *Space Sci. Rev.* **58**, 1–185 (1991).
10. Sittler, E. C. Jr. & Strobel, D. F. *J. geophys. Res.* **92**, 5741–5762 (1987).
11. Bagenal, F. J. *geophys. Res.* **99**, 11043–11062 (1994).
12. Zipf, E. C. *J. Phys. B* **19**, 2199–2209 (1986).
13. Itikawa, Y. et al. *J. Phys. Chem. Ref. Data* **18**, 23–42 (1989).
14. Laher, R. R. & Gilmore, F. R. *J. Phys. Chem. Ref. Data* **19**, 277–305 (1990).
15. Shemansky, D. E. *J. geophys. Res.* **93**, 1773–1784 (1988).
16. Lee, J. S. & Meier, R. R. *Astrophys. J.* **240**, 185–195 (1980).
17. Gladstone, G. R. *J. geophys. Res.* **97**, 19519–19525 (1992).
18. Meier, R. R. & Lee, J. S. *Planet. Space. Sci.* **30**, 439–450 (1982).
19. Johnson, R. E. et al. *geophys. Res. Lett.* **10**, 892–895 (1983).
20. Cheng, A. F., Haff, P. K., Johnson, R. E. & Lanzerotti, L. J. in *Satellites* (eds Burns, J. A. & Matthews, M. S.) 403–436 (Univ. Arizona Press, Tucson, 1986).

ACKNOWLEDGEMENTS. We thank R. R. Meier for calculations confirming the accuracy of equation (4), and D. E. Shemansky for providing program COREQ to calculate O⁺ electron-impact excitation rates. This work was supported by NASA through a grant from the Space Telescope Science Institute, which is operated by the Association of Universities for Research.

High current densities above 100 K in the high-temperature superconductor HgBa₂CaCu₂O_{6+δ}

L. Krusin-Elbaum, C. C. Tsuei & A. Gupta

IBM T. J. Watson Research Center, Yorktown Heights, New York 10598-0218, USA

THE recent discovery^{1,2} of a family of mercury-based copper oxide superconductors having transition temperatures above 130 K is of considerable technological interest. But the viability of high-temperature superconductors for many applications will ultimately depend on the size of the current density, J_c , that they are able to support, not only at high temperatures, but also in high magnetic fields. For the cuprate superconductors, and in particular for Hg-based materials, the combination of high transition temperature³ and large mass anisotropy implies that the transport properties will be intrinsically limited by large thermal fluctuations and short superconducting coherence lengths⁴. Here we report that high-quality *c*-axis-oriented epitaxial films of the compound HgBa₂CaCu₂O_{6+δ} (Hg-1212; ref. 5) can support large in-plane current densities at temperatures higher than has been achieved for other superconductors. In low magnetic fields oriented normal to the film surface, we find $J_c \geq 10^7$ A cm⁻² at 5 K and $J_c \sim 10^5$ A cm⁻² at 110 K, at least an order of magnitude larger than for Bi- or Tl-based films^{6–11}. For in-plane magnetic fields, the critical current ($\sim 10^8$ A cm⁻²) is close to the theoretical limit even at high fields, indicative of strong intrinsic pinning in this compound.

The procedure for synthesis of Hg-1212 films from precursor oxides has been described in detail elsewhere⁵. The sequential

layers of HgO and the copper oxide precursor Ba₂CaCu₂O_x were deposited from two separate targets by a pulsed laser technique. The films were deposited at room temperature *in vacuo* on (100)-oriented SrTiO₃ substrates. In this study we used 0.25-μm-thick films which were covered by a protective layer of HgO (400 Å thick). The films were then annealed at 800 °C for 1 h in sealed quartz tubes together with appropriate amounts of bulk stoichiometric mercury copper oxide and pellets of the precursor. This combination was chosen to provide an optimal Hg atmosphere and O₂ partial pressure consistent with thermodynamic requirements for stabilizing the Hg-1212 phase at the annealing temperature⁵.

Such annealed films have a sharp resistive transition and equally sharp onset of diamagnetism at the same temperature, as shown in Fig. 1 for a typical film with superconducting transition temperature T_c of ~ 120 K. Figure 2 shows the temperature dependence of persistent current for the film of Fig. 1 for three values of magnetic field applied normal to the plane of the film. The persistent current density was obtained from the width of direct current magnetic hysteresis $\Delta M = \Delta m/V$ using the critical-state model¹², which relates ΔM to J via a geometrical factor. Here, $m(\equiv m(H, T))$ where H is magnetic field strength and T is temperature) is the magnetic moment we measure with a superconducting quantum interference device magnetometer and V is the volume of the superconductor. This geometrical conversion depends on current anisotropy¹³ and on the current loop dimension, which may be limited by the grain size. Indeed, in bulk ceramics, the critical current density J_c can be reduced by the formation of weak links at the grain boundaries¹⁴, although such weak-link behaviour can be substantially eliminated in thin films^{6–11}. For small in-plane anisotropy (that is, $J^a \approx J^b = J^{ab}$), we adopt the notation $J^{ab,c}$ for the currents in the *ab*-plane and H parallel to the *c*-axis. At low fields and at low temperatures (5 K), $J^{ab,c} \approx 10^7$ A cm⁻². (For comparison, the 5 K transport $J_c^{ab,c}$ for excellent-quality films of Bi-2223 is⁶ $\sim 2 \times 10^6$ A cm⁻².) Here we have taken the size of the sample as the relevant loop dimension, as the current density is high and we verified that ΔM scales with the size of the sample. The initial small drop of J at low fields indicates the presence of some weak links (for

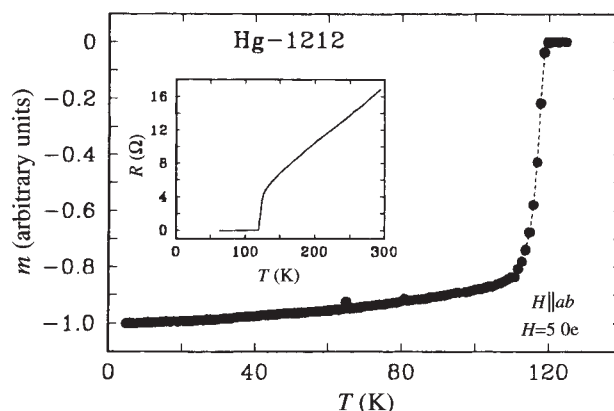


FIG. 1 A typical diamagnetic transition for a 0.25-μm-thick Hg-1212 film in an in-plane magnetic field of 5 Oe (m is defined in the text). In this orientation (H parallel to the *ab*-plane) the demagnetization effect is small and transition at temperature $T = 120$ K is very sharp. Inset, the zero-field resistive transition for the same film. All films gave sharp X-ray diffraction patterns of pure Hg-1212, with the *c*-axis (lattice parameter $d = 12.54$ Å) aligned normal to the plane of the film, although small amounts of single-layer Hg-1201, present as randomly distributed surface precipitates, were also detected. The stoichiometry was ensured by calibrating the deposited films with Rutherford backscattering.

example, high-angle grain boundaries), but it is less pronounced than in bismuth-based epitaxial films^{6,7}. As the temperature T is increased, J decreases nearly exponentially as $\sim \exp(-T/T_0)$, indicative of flux creep¹⁵ (thermally activated motion of magnetic vortices⁴). Remarkably, though, at low fields even at 110 K, $J^{ab,c}$ (a lower bound for the critical current density $J_c^{ab,c}$) is $\sim 10^5$ A cm⁻², the highest value measured magnetically at this temperature. Indeed, the self-field transport $J_c^{ab,c}$ obtained on a similar film¹⁶ (also shown in Fig. 2) is $\sim 6 \times 10^5$ A cm⁻² at 110 K, over an order of magnitude higher than was reported in TI-based films at this temperature⁹⁻¹¹, and consistent with creep-reduced magnetic J at high temperatures.

At high fields, $J^{ab,c}$ declines precipitously owing to a rather low-lying irreversibility line for H at right angles to the CuO₂ planes. The irreversibility line $H_{irr}(T)$ is a boundary in the field-temperature (H - T) plane which separates the useful regime of pinned vortices (with finite J_c albeit affected by creep) and the regime of mobile vortices (with $J_c \approx 0$). Figure 3 shows $H_{irr}(T)$ obtained from the pinch-off of the magnetic hysteresis (with the criterion of $\sim 10^{-5}$ e.m.u.) for both H parallel to the c -axis and parallel to the ab -planes. In the former case, $H_{irr}(T)$ is only slightly above that of crystalline Bi-2212^{17,18}, indicative of large anisotropy⁴. For H parallel to the ab -plane, there is an important issue of possible misalignment, although the different shapes of $H_{irr}(T)$ for the two field orientations prevent the expected scaling by the cosine of the angle between the field and the c -axis^{8,19}.

For a layered material, the flux line lattice is expected to be of a quasi-two-dimensional type at sufficiently high fields^{4,19} $H \geq B^{2D} \approx 4\Phi_0/\Gamma s^2$, where Φ_0 is the flux quantum, s is the spacing between the double CuO₂ layers, and Γ is the effective mass anisotropy. The melting line of vortices above B^{2D} is exponential in temperature²⁰, $B(T) \approx B^{2D} \exp(T_m^{2D}/T)$, where T_m^{2D} is the field-independent 2D melting temperature of the vortex array. If we take our H_{irr} for H parallel to the c -axis at high fields as an approximation to the melting line, we can extract an estimate of B^{2D} , and hence a rough estimate of the anisotropy parameter Γ . The plot of $\ln H_{irr}$ versus inverse temperature is shown in Fig. 3 inset. From the intercept and with $s \sim 11$ Å, we obtain $B^{2D} \approx 1.3$ T and $\Gamma \approx 4,500$, within the range of values reported^{21,22} for Bi-2212.

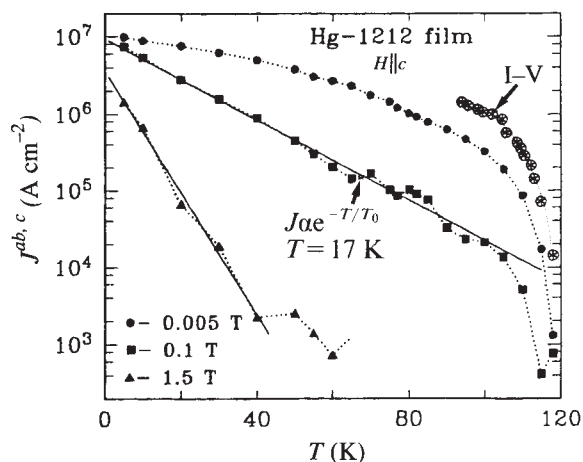


FIG. 2 Temperature dependence of the in-plane persistent current density $J^{ab,c}$ for H normal to the film surface. $J^{ab,c}(T, H)$ falls off quasi-exponentially with temperature due to flux creep, and decreases rapidly in high fields. The value of the empirical parameter T_0 (see text) at 0.1 T (17 K) is comparable to that of Y-123 (YBa₂Cu₃O_{7- δ}) (where $T_0 \approx 23$ K), but drops to a value closer to Bi-2212 in a 1.5-T field¹⁵. The self-field transport $J_c^{ab,c}$ obtained with $1 \mu\text{V mm}^{-1}$ voltage criterion is shown as encircled stars.

Generally, estimating current densities for H parallel to the ab -plane from the irreversible magnetization $M_{ab}(H)$ is quite complicated. The currents flow across the CuO₂ layers ($J^{c,ab}$) as well as in the layers ($J^{ab,ab}$), and both contribute to the irreversible magnetization¹³. Because the current anisotropy may be significant, the anisotropic extension of the critical-state model¹² should be used and we proceed here with an estimate following Gyorgy *et al.*¹³. In general, to separate the contributions from the two currents one should measure M_{ab} for vastly different aspect ratios t/ℓ , where t is the film thickness and ℓ is the width of the sample. For high-aspect ratio crystalline samples ($t/\ell \approx 1$), the irreversible magnetization M_{ab} will be dominated by the weaker current $J^{c,ab}$. However, in our thin-film samples, the aspect ratio is very low (here $t = 2.5 \times 10^{-5}$ cm and $\ell = 0.45$ cm). Still, the issue is the value of the c -axis current $J^{c,ab}$. In Bi-2212 this current is²³ only ~ 1000 A cm⁻² at 4.2 K; this is only a few times smaller than the large-angle (37°) grain-boundary current J^{gb} , also at 4.2 K, in this material¹¹. The J^{gb} is reduced¹¹ by a factor of ~ 5 when the temperature is increased to $\sim 0.6T_c$. In the following calculation, we will take J^{gb} as an order-of-magnitude estimate for $J^{c,ab}$. We do know the value of the 37° grain-boundary current for our films¹⁶; it is $\sim 10^4$ A cm⁻² at 70 K—scaled-up by over an order of magnitude from that in Bi-2212. Within this estimate, the magnetic hysteresis will be controlled by the in-plane current component¹³, that is $40\Delta M_{ab}/t \approx J^{ab,ab}$. Thus we can attempt a crude estimate of the in-plane current density with the applied field also in the ab -plane. The temperature variation of $J^{ab,ab}$ is shown in Fig. 4 for $H > 1$ T. We obtain remarkably high values of $J^{ab,ab}$; $\sim 10^8$ A cm⁻² at (and below) 1 T, near the limit of depairing, at which the kinetic energy of the superconducting carriers equals the condensation energy $H_c^2(0)/8\pi$, where H_c is the thermodynamic critical field. This is the ultimate limit allowed by the BCS theory. Indeed, taking coherence length $\xi \approx 20$ Å and the magnetic penetration depth $\lambda \approx 1,170f$ Å (ref. 24), we estimate the depairing current density $J_0 = 4/3\sqrt{3}(c/\xi)(\epsilon_0/\Phi_0) \approx 2 \times 10^8$ A cm⁻², where c is the velocity of light and $\epsilon_0 = (\Phi_0/4\pi\lambda)^2$ is the basic energy scale⁴. Although the above estimates are tentative, the alternative is that $J^{c,ab}$ is lower than J^{gb} . A rough estimate¹³ gives $J^{c,ab} \approx 5,000$ A cm⁻², with the expectation of a very strong (decreasing with increasing field) field dependence. The latter is not borne out by the experiment (Fig. 4 inset), which shows a very weak field dependence below the irreversibility line⁸.

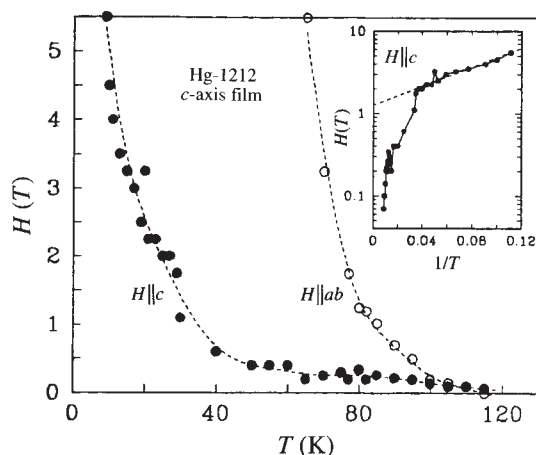


FIG. 3 Irreversibility lines of Hg-1212 c -axis-oriented film for H parallel to the c -axis and for H parallel to the ab -planes; both were obtained from the closing of the hysteresis loops $m(H)$ at different temperatures with the criterion of $\sim 10^{-5}$ e.m.u. For H parallel to the c -axis, $H_{irr}(T)$ is very similar to the value^{14,17,18} for Bi-2212. Inset, semi-log plot of $H_{irr}(T)$ versus inverse temperature.

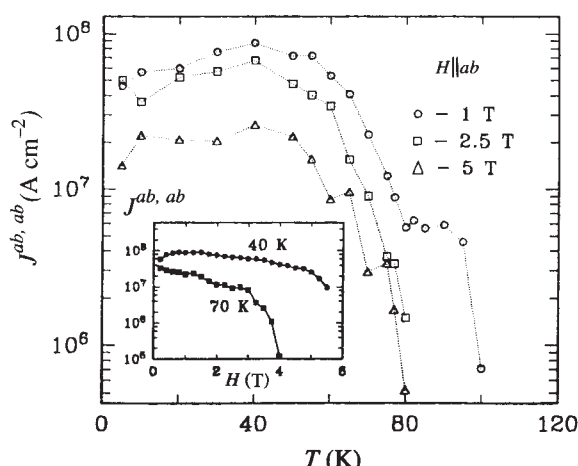


FIG. 4 Temperature dependence of the in-plane persistent current density $J^{ab,ab}$ for the in-plane H perpendicular to J . $J^{ab,ab}$ is only weakly field dependent and approaches the depairing current of $\sim 10^8 \text{ A cm}^{-2}$ at lower fields. This behaviour is indicative of strong intrinsic pinning by the layered structure itself.

The high values of $J^{ab,ab}$ can be accounted for by intrinsic pinning²⁵. Because of the large spacing between the double CuO_2 layers ($\sim 11\text{--}12 \text{ \AA}$), the superconducting order parameter should be significantly reduced in these regions and thus it is energetically favourable for vortices to lie between the layers. For the in-plane fields in our configuration, the Lorentz force is along the c -axis and there is a large energy barrier for moving long segments of vortices across the CuO_2 layers; this barrier is controlled by the crystal lattice and the intrinsic anisotropy of the material. In such a configuration, special precautions must be

taken to align the film; otherwise, the contribution of H parallel to the c -axis may not be negligible. Our films were gravity-aligned; we have carefully attached our samples on a varnish-stiffened string which was weighted down with a massive platinum cylinder. An alignment of better than $\sim 0.5^\circ$ is, however, extremely difficult to achieve and undoubtedly we see the effect of some misalignment in $H_{irr}(T)$. The weak field-dependence of $J^{ab,ab}$ is indeed characteristic of strong intrinsic pinning in Hg-1212 films. □

Received 23 September 1994; accepted 17 January 1995.

1. Putlin, S. N., Antipov, E. V., Chmaissem, O. & Marezio, M. *Nature* **362**, 226–228 (1993).
2. Schilling, A., Cantoni, M., Guo, J. D. & Ott, H. R. *Nature* **363**, 56–58 (1993).
3. Meng, R. L. et al. *Physica C* **214**, 307–312 (1993).
4. Blatter, G. et al. *Rev. mod. Phys.* (in the press).
5. Tsuei, C. C., Gupta, A., Trafas, G. & Mitzi, D. *Science* **263**, 1259–1261 (1994).
6. Endo, K., Yamasaki, H., Misawa, S., Yoshida, S. & Kajimura, K. *Nature* **355**, 327–328 (1992).
7. Yamasaki, H. et al. *Phys. Rev. Lett.* **70**, 3331–3334 (1993).
8. Schmitt, P., Kummeth, P., Schultz, L. & Saemann-Ischenko, G. *Phys. Rev. Lett.* **67**, 267–270 (1991).
9. Chu, M. L. et al. *Appl. Phys. Lett.* **59**, 1123–1125 (1991).
10. Ren, Z. F., Wang, C. A. & Wang, J. H. *Appl. Phys. Lett.* **65**, 237–239 (1994).
11. Kawasaki, M. et al. *Appl. Phys. Lett.* **62**, 417–419 (1993).
12. Campbell, A. M. & Evetts, J. E. *Adv. Phys.* **21**, 199–429 (1972).
13. Gyorgy, E. M., van Dover, R. B., Jackson, K. A., Schneemeyer, L. F. & Waszczak, J. V. *Appl. Phys. Lett.* **55**, 283–285 (1989).
14. Umezawa, A. et al. *Nature* **364**, 129–131 (1993).
15. Christen, D. K. & Thompson, J. R. *Nature* **364**, 98–99 (1993).
16. Gupta, A., Sun, J. Z. & Tsuei, C. C. *Science* **265**, 1075–1077 (1994).
17. Huang, Z. J., Xue, Y. Y., Meng, R. L. & Chu, C. W. *Phys. Rev. B* **49**, 4218–4221 (1994).
18. Welp, U. et al. *Appl. Phys. Lett.* **63**, 693–695 (1993).
19. Kes, P. H., Aarts, J., Vinokur, V. M. & van der Beek, C. J. *Phys. Rev. Lett.* **64**, 1063–1066 (1990).
20. Feigel'man, M. V., Geshkenbein, V. B. & Larkin, A. I. *Physica C* **167**, 177–187 (1990).
21. Farrell, D. E. et al. *Phys. Rev. Lett.* **63**, 782–785 (1989).
22. Martinez, J. C. et al. *Phys. Rev. Lett.* **69**, 2276–2279 (1992).
23. Kleiner, R. & Müller, P. *Phys. Rev. B* **49**, 1327–1341 (1994).
24. Thompson, J. R. et al. *Phys. Rev. B* **48**, 14031–14034 (1993).
25. Tachiki, M. & Takahashi, S. *Solid St. Commun.* **70**, 291–295 (1989).

ACKNOWLEDGEMENTS. We are pleased to acknowledge valuable discussions with L. Bulayevskii, J. R. Clem, W. J. Gallagher and J. R. Thompson.

Differences in the chemical reactivity of individual molecules of an enzyme

Qifeng Xue & Edward S. Yeung*

Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, Iowa 50011, USA

MUCH attention has been focused recently on the detection and physical characterization of individual molecules^{1–11}. Using such methods to study the chemical properties, such as reactivity, of single molecules offers the potential to investigate how these might vary from molecule to molecule, and for individual molecules as a function of time. The complex structures of biomolecules such as enzymes make them particularly attractive targets for studying how subtle changes or differences at the molecular level might influence chemical reactivity. We have shown previously^{12,13} that very small (zeptomole) amounts of enzymes can be studied using a fluorescence microassay; single enzyme molecules have also been detected in oil-dispersed droplets by fluorescence microscopy^{14,15}. Here we report the observation of reactions of individual molecules of lactate dehydrogenase (LDH-1), which produces NADH from lactate and nicotinamide adenine dinucleotide (NAD^+). When they are present at very low concentrations in a narrow capillary, each

enzyme molecule produces a discrete zone of NADH; these can be manipulated electrophoretically and monitored by fluorescence spectroscopy. We find that the activity of individual electrophoretically pure enzyme molecules can vary by up to a factor of four, and that these activities remain unchanged over a two-hour period. We suggest that the origin of the activity differences may lie in the presence of several stable forms of the enzyme.

The experimental apparatus was adapted from that described in ref. 12. At pH 9.1, LDH-1 shows maximum activity for catalysing lactate to pyruvate conversion, and NADH shows higher fluorescence efficiency compared to that at pH 7. By increasing the incubation temperature from 21 to 40 °C, the reaction rate is increased without denaturing the enzyme¹⁶. NADH left in the capillary for 1 h does not show serious axial broadening (diffusion coefficient $D \sim 10^{-5} \text{ cm}^2 \text{ s}^{-1}$), even though it will be well mixed radially. So we are able to increase the incubation period, relative to ref. 12, from 2 min to 1 h; this enhances the sensitivity of detection, as more NADH is produced per molecule of enzyme. In all, we have improved the previous¹² detection limit for LDH-1 by a factor of 10^3 .

To avoid contamination, all sample solutions were carefully filtered (0.22 μm). A $7.6 \times 10^{-6} \text{ M}$ enzyme solution was diluted in three steps, so that only a few molecules of enzyme existed in the capillary when it was filled. The activity of each of these molecules can be monitored individually by driving each product zone electrophoretically past an argon-ion laser (which excites a region 10 μm in width). Each enzyme molecule is followed by a product zone of NADH, the concentration of which (typically containing $\sim 2 \times 10^7$ molecules of NADH) is determined by the activity of the enzyme molecule. Fluorescence from the NADH,

* To whom correspondence should be addressed.

at a wavelength of 460 nm, is monitored by a photomultiplier, via a $\times 20$ microscope objective lens. The intensity of fluorescence thereby provides an indication of the enzyme's activity (Fig. 1). When the capillary was re-filled with the substrates only, immediately after an enzyme assay, incubation for the same period did not produce any recognizable peaks, showing that memory effects owing to adsorption and other artefacts are negligible.

The numbers of LDH-1 molecules that exist in the capillary (290 nl volume) are predicted to be 15 and 30, respectively, for the concentrations of 7.6×10^{-17} and 1.5×10^{-16} M. The average numbers of peaks found were 8 ± 2 and 13 ± 1 (ten separate experiments) respectively, both less than calculated but in a 1:2 ratio. This is partly due to difficulties in preparing precisely very dilute solutions. Furthermore, the commercial enzyme preparation is not free of inactive molecules or other proteins. The individual peak areas (average, 343 units), which are independent of axial diffusion, are also consistent with experiments performed at high LDH-1 concentrations when extrapolated to single molecules (308 units).

The width of the individual peaks in Fig. 1 are nearly identical, as would be expected from the time available for axial diffusion. The feature at 2.4 min is unusually broad, indicating possible overlap of two product zones. The probability that any zone is due to two enzyme molecules residing in the exact region is given by the ratio between the zone length and the capillary length, which is $\sim 1/30$. We would predict that ~ 3 in the set of 79 are double-molecule zones. In Fig. 2 there are indeed three peaks ($>1,000$ units) that are much larger than the others. Unfortunately, these cannot be independently verified as double-molecule zones. The relative standard deviation (RSD) of the activity distribution is 69% for 79 clearly isolated peaks. If the three questionable peaks are excluded, the RSD becomes 46%, and the revised average area of the plot (306 units) agrees even better with the extrapolated value from high-concentration experiments. The RSD for areas in single runs ranged from 32% to 72%, showing that the variations are characteristic of individual molecular properties rather than differences in reaction conditions. About 2×10^7 NADH molecules are produced in each zone, so fluctuations in substrate concentration around each molecule cannot be responsible for these variations.

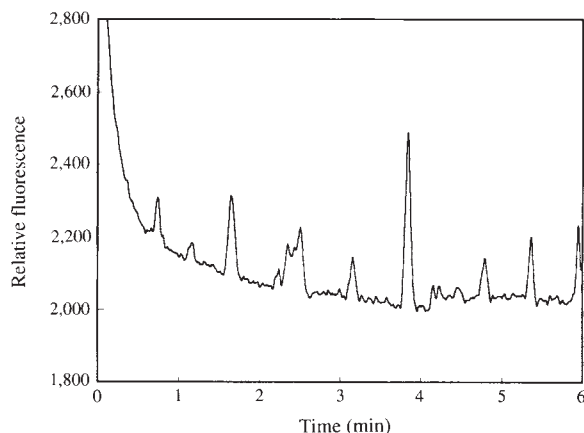


FIG. 1 Detection of single LDH-1 molecules. A LDH-1 solution (7.6×10^{-17} M) was incubated at 40 °C in a 20- μ m-diameter capillary tube together with 1 mM NAD⁺, 3 mM lactate and 20 mM Tris buffer (pH 9.1) for 1 h. Fluorescence at 460 nm was then collected by a $\times 20$ microscope objective and recorded by a phototube as the individual NADH zones created with the 70-cm section were electrophoretically driven at 11 cm min⁻¹ (by an applied voltage of 24 kV) through a 50-mW argon-ion laser beam (305 nm wavelength), formed into a 10- μ m spot.

It should be noted that we have actually determined the concentration of molecules in a solution at 10^{-17} M, because the total count and the total volume are known. The precision is limited by molecule-counting statistics, and can be improved by using longer capillaries. Previous reports involving single-molecule detection^{8,17} are based on samples at 10^{-14} M or higher concentrations. Furthermore, the molecules here need not be fluorescent or pretreated in any way, because the product of a specific reaction is monitored.

We can monitor the activity of individual molecules as a function of time, by electrophoretically separating each LDH-1 molecule from its first NADH product zone, incubating for another hour, and then measuring the fluorescence from the second product zone. Such observations are depicted in Fig. 3, where we see pairs of peaks separated by an interval of ~ 0.12 – 0.15 min. This separation is comparable to that predicted (0.14 min) from the application of electrophoresis for 3 min, based on the known mobilities of LDH-1 and NADH. The fact that a second peak is seen at all indicates that our observations are indeed due to enzyme reactions and not impurities, and that adsorption at the walls is negligible. The first peak is always broader than the second, because the NADH has had longer in this case to diffuse axially. The peak widths are narrower than those in Fig. 1, which is consistent with viscosity differences at the two temperatures. Also, the peak areas in Fig. 1 are 3–4 times larger than those in Fig. 3, as predicted from typical activation energies within this temperature range.

Most importantly, the intensities of the pairs of peaks in Fig. 3 remain constant, with an average ratio for 18 molecules of 1.03 ± 0.11 . Clearly, the reactivities are not influenced by the local environment, such as wall interactions. This also shows that the differences in reactivities among LDH-1 molecules, which can be as large as a factor of four, are real. This constancy of LDH-1 activity over a two-hour period is consistent with expectation¹⁸.

Differences in activities amongst individual molecules can best be explained by specific stable conformational arrangements of the four identical subunits¹⁹, making certain active sites²⁰ less accessible than others for reaction. Indeed, reassociation of LDH at similar temperatures after denaturation is known to lead to structural variants that exhibit different physical and chemical properties²¹, enzyme activity²² and spectroscopic properties²³.

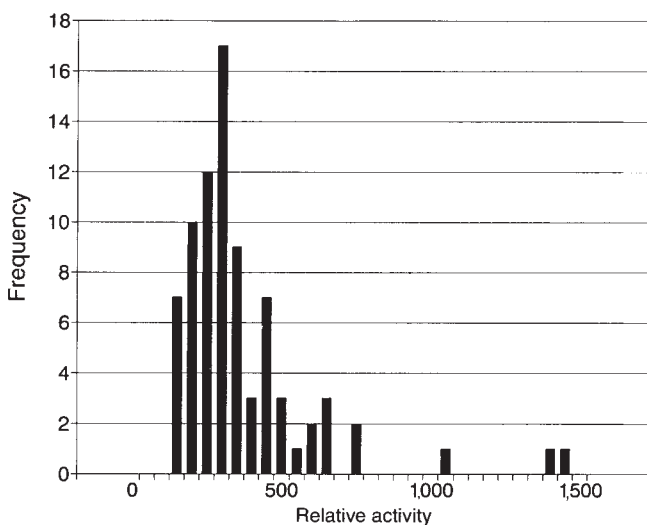


FIG. 2 Histogram of single-molecule reactivities. The background-normalized peak area in Fig. 1 is proportional to the activity of individual LDH-1 molecules, as the presence of excess substrates ensures a pseudo-first-order reaction.

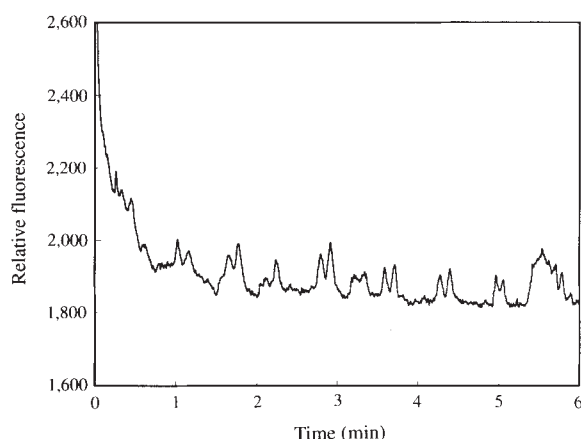


FIG. 3 The activity change of individual LDH-1 molecules with time at 21 °C. Two one-hour incubation periods separated by electrophoresis for 3 min were employed in the experiment, creating pairs of peaks. The solution concentration of LDH-1 was 4.5×10^{-17} M. Other conditions were as in Fig. 1 except that a 75-cm section of capillary tube was monitored.

The continuous distribution of peak areas (Fig. 2) indicates the presence of several conformers.

This concept can be extended to any species that can catalyse the production of a suitable fluorophore. With the appropriate linkage of LDH-1 to a hybridization tag or to an antibody, practically any molecule can be counted in this way. Each

molecule can be characterized, not just counted. The protocol described here also demonstrates how single molecules can be selected and collected in a small volume, for example in diagnostics and in biotechnology¹⁷, because one can extrapolate from the electrophoretic mobilities when each molecule will elute out of the capillary. □

Received 16 September 1994; accepted 17 January 1995.

- Moerner, W. E. *Science* **265**, 46–53 (1994).
- Oritt, M., Bernard, J. & Personov, R. I. *J. phys. Chem.* **97**, 10256–10268 (1993).
- Betzig, E. & Chichester, R. J. *Science* **262**, 1422–1425 (1993).
- Ambrose, W. P., Goodwin, P. M., Martin, J. C. & Keller, R. A. *Science* **265**, 364–367 (1994).
- Xie, X. S. & Dunn, R. C. *Science* **265**, 361–364 (1994).
- Ishikawa, M., Hirano, K., Hayakawa, T., Hosoi, S. & Brenner, S. *J. appl. Phys.* **33**, 1571–1576 (1994).
- Smith, R. D., Cheng, X., Bruce, J. E., Hofstadler, S. A. & Anderson, G. A. *Nature* **369**, 137–139 (1994).
- Barnes, M. D., Ng, K. C., Whitten, W. B. & Ramsey, J. M. *Analyt. Chem.* **65**, 2360–2365 (1993).
- Smith, S. B., Aldridge, P. K. & Callis, J. B. *Science* **243**, 203–206 (1989).
- Perkins, T. T., Smith, D. E. & Chu, S. *Science* **264**, 819–822 (1994).
- Bezrukov, S. M., Vodyanoy, I. & Parsegian, V. A. *Nature* **370**, 279–281 (1994).
- Xue, Q. & Yeung, E. S. *Analyt. Chem.* **66**, 1175–1178 (1994).
- Miller, K. J., Leesong, I., Bao, J., Regnier, F. E. & Lytle, F. E. *Analyt. Chem.* **65**, 3267–3270 (1993).
- Rotman, B. *Proc. natn. Acad. Sci. U.S.A.* **47**, 1981–1991 (1961).
- Rotman, B. in *Fluorescence Techniques in Cell Biology* (eds Thae, A. A. & Sernetz, M.) 333–337 (Springer, New York, 1973).
- Stranford, P. E., Clayton, K. J. & Freier, E. F. *J. Am. med. Ass.* **182**, 123–126 (1962).
- Eigen, M. & Rigler, R. *Proc. Natn. Acad. Sci. U.S.A.* **91**, 5740–5747 (1994).
- Zubay, G. *Biochemistry* 3rd edn 859 (Brown, Dubuque, Iowa, 1993).
- Everse, J. & Kaplan, N. O. *Adv. Enzymol.* **37**, 61–133 (1973).
- Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossmann, M. G. in *The Enzymes* 3rd edn Vol. II 280–281 (Academic, New York, 1975).
- Levi, A. S. & Kaplan, N. O. *J. biol. Chem.* **246**, 6409–6417 (1971).
- Teipel, J. W. & Koshland, D. E. *Biochemistry* **10**, 792–798 (1971).
- Teipel, J. W. & Koshland, D. E. *Biochemistry* **10**, 798–805 (1971).

ACKNOWLEDGEMENTS. The Ames Laboratory is operated for the US Department of Energy by Iowa State University. This work was supported by the US Office of Basic Energy Sciences.

A possible prebiotic synthesis of pantetheine, a precursor to coenzyme A

Anthony D. Keefe, Gerald L. Newton & Stanley L. Miller

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093, USA

THE involvement of coenzyme A in many enzyme reactions suggests that it acted in this capacity very early in the development of life on Earth. Particularly relevant in this regard is its role in the activation of amino acids and hydroxy acids in the biosynthesis of some peptide antibiotics^{1,2}—a mechanism of peptide synthesis that forms the basis for the proposal that a thioester world³ could have preceded the RNA world⁴. The components of coenzyme A have been shown to be probable prebiotic compounds: β -alanine, pantoyl lactone and cysteamine^{5,6} and possibly adenosine^{7,8}. We show here that the pantetheine moiety of coenzyme A (which also occurs in a number of enzymes) can be synthesized in yields of several per cent by heating pantoyl lactone, β -alanine and cysteamine at temperatures as low as 40 °C. These components are extremely soluble and so would have been preferentially concentrated in evaporating bodies of water, for example on beaches and at lagoon margins. Our results show that amide bonds can be formed at temperatures as low as 40 °C, and provide circumstantial support for the suggestion that pantetheine and coenzyme A were important in the earliest metabolic systems.

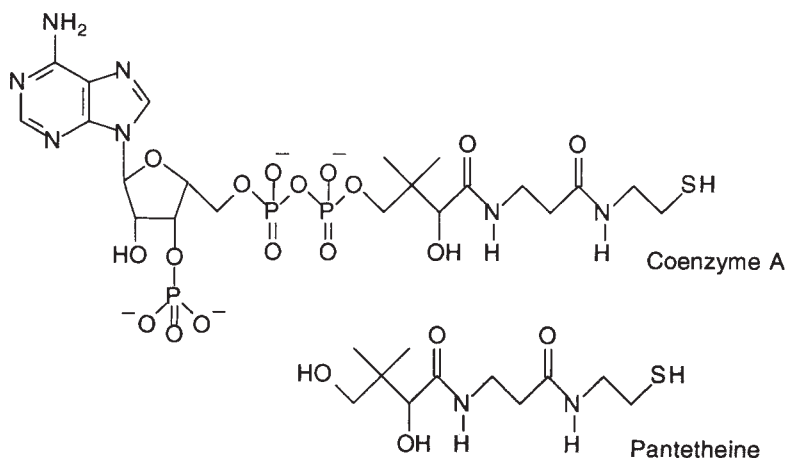
The complexity of coenzyme A (Fig. 1) is puzzling, as almost any thiol would be able to perform its function of activating

carboxyl groups. One possibility⁶ is that pantetheine was simply formed rather easily in the prebiotic world. Pantetheine is essentially coenzyme A without the adenosine diphosphate group attached (Fig. 1). It is used in a number of enzymes to which it is attached via a serine phosphate¹, and is plausibly a precursor to coenzyme A. Possible prebiotic routes to all of the components of pantetheine can be postulated. β -Alanine is efficiently synthesized prebiotically^{9,10} and occurs in the Murchison meteorite^{11,12}. Cysteamine is likely to have been one of the most abundant thiols in the primitive ocean as it is easily synthesized from ethylene sulphide and ammonia, (or from ethylene imine and H₂S). The most likely prebiotic source of ethylene sulphide is by reaction of a sulphur atom with ethylene¹³. The dihydroxy acid pantonic acid is synthesized by the addition of formaldehyde and HCN to isobutyraldehyde or α ketoisovaleric acid⁶. Pantothenic acid is formed from pantoyl lactone and β -alanine under prebiotic conditions⁶. Here we show that cysteamine can also be incorporated to produce pantetheine.

Solutions containing 1 mmol each of pantoyl lactone, β -alanine and cysteamine were adjusted to pH 7.0, placed in glass vials and evaporated at 25 °C *in vacuo*. The vials were then evacuated, sealed and then heated in a heating block. This procedure was adopted to allow the reaction to proceed in the absence of an oxidizing atmosphere. Relatively large samples were used as the yield decreased with sample size reduction. This is possibly due to reaction with traces of oxygen or to inadequate mixing of the components within the resultant thin film.

The heated samples were initially reacted with monobromobimane to give fluorescent species which were then separated by high-performance liquid chromatography (HPLC)¹⁴. The peaks were identified by comparison of their retention times with those of a known sample of the pantetheine derivative. This was followed by the spiking of each sample with the known compound, and the mixture was again separated by HPLC. The pantetheine

FIG. 1 Structures of coenzyme A and pantetheine.



identification was then confirmed by proton NMR (300 MHz, G.E. Model No. QE-300) of the derivitized unknown purified by HPLC and comparison with an authentic sample (Fig. 2). Figure 3 shows the yield of pantetheine as a function of time for 60, 80, 100 and 118 °C. At 118 and 100 °C, the yield rises rapidly and then levels off at ~1%. At 80 °C the curve is still rising after 120 h, and at 60 °C it is still rising after 6 weeks. The 40 °C curve is not shown, but at 1 month the yield was measured as 0.018% and after 2 months as 0.034%. The yield of pantetheine is surprisingly high considering the large number of other

possible products. Temperatures of 65 °C are observed in desert sands at present¹⁵ and so these reactions may have occurred extensively on the primitive Earth.

The rate of synthesis calculated from the yields of the first few points is shown in an Arrhenius plot in Fig. 4. The plot is linear and gives an activation energy of 25.8 kcal. Surprisingly there is no threshold for the reaction, which may be due to the components not being solids, but rather dissolved in a very concentrated aqueous solution. Attempts to completely dessicate the reaction mixture in a vacuum desiccator over sodium hydroxide pellets at room temperature were unsuccessful. This would also presumably have been the case on a dried lagoon or beach. The importance of the humidity has been demonstrated in prebiotic phosphorylation reactions¹⁶.

There have been extensive prebiotic experiments on the polypeptides produced¹⁷ by heating mixtures of amino acids at 150–180 °C. These temperatures were extremely rare on the prebiotic Earth and in any case they would have caused the rapid destruction of the amino acids. A few experiments have been carried out at lower temperatures, but the products were not shown to be peptides¹⁸. Our experiments at 40 °C are at the lowest temperature used so far in which amide bonds are formed by dry heating and produce an identified compound. At such a low temperature the rate of destruction of organic compounds is extremely slow compared to the rate at 150–180 °C.

The problem of concentrating and reacting the components of coenzyme A in the presence of many other prebiotic molecules needs to be considered. Reported yields are for mixtures of reactants in the absence of other species. On the prebiotic Earth, in the absence of efficient separation mechanisms for most species, there would have been very many compounds present in the ocean. Reactions under these conditions would have usually resulted in many products and a consequent reduction in the yield of any particular product. An increase in yields of products would have resulted from segregation of desired reactants.

In this particular example the proposed precursors may be easily separated and concentrated on the grounds of solubility; β -alanine is one of the most soluble amino acids¹⁹ (9.85 molal, 47 wt% at 25 °C). The solubilities of pantoyl lactone and cysteamine hydrochloride were measured by preparing saturated solutions of each compound in a rotating sealed glass vial maintained by thermostatic bath at 25 °C. A sample of the solution was accurately weighed, diluted and titrated with NaOH. The solubilities were also measured using ¹H NMR. A spectrum was taken of a sample of the solution diluted with perdeuterio-dimethyl sulphoxide, and the ratio of the intensities of the water and the organic peaks was used to calculate the solubility; this method is only suitable for extremely soluble compounds. A saturated solution of pantoyl lactone is 94 wt% (120 molal) at

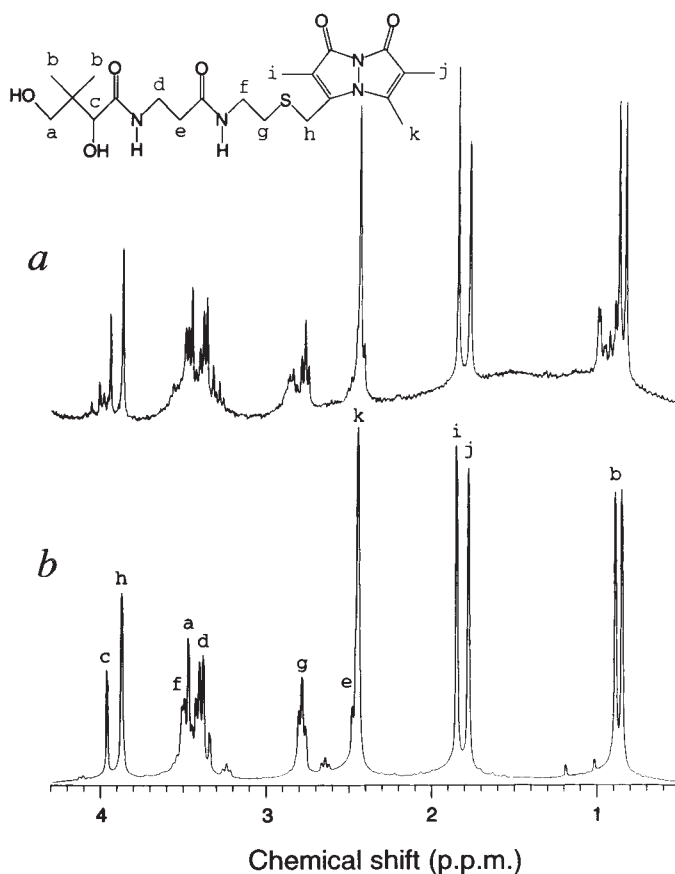


FIG. 2 ¹H NMR spectra (300 MHz, G.E. Model No. QE-300) in D₂O of unknown (a) and known (b) pantetheine bimeane derivatives after purification by HPLC. The assigned peaks are indicated on the formula. The unassigned peaks are impurities.

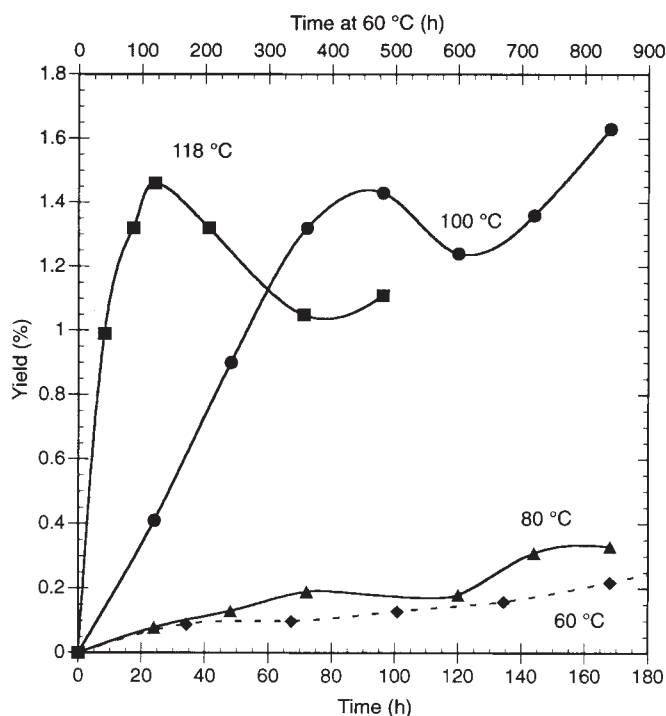


FIG. 3 The yields of pantetheine as a function of time with the 60 °C plot having the timescale reduced by a factor of five. One mmol each of pantoyl lactone, β -alanine and cysteamine were dissolved in 2 cm³ of deionized water and the pH of the solution was adjusted to 7.0. Solutions were evaporated and sealed under vacuum in glass vials. After heating, the samples were derivitized with monobromobimane and chromatographed on an Altex Ultrasphere octadecyl silane 5 μ m column with a fluorescence detector¹⁵. The pH of a solution of the heated samples is 4.0–4.1.

25 °C, and cysteamine hydrochloride is 86 wt% (54 molal) at 25 °C. The last part of a sample of primitive ocean water to evaporate in a lagoon would have been greatly enriched in these components of pantetheine. This would have provided an ideal environment for the chemistry we have described above. The high solubility of the components of pantetheine together with

their prebiotic abundance offers a rational explanation for the presence of β -alanine, pantoyl lactone and cysteamine in the first thioester-forming coenzyme.

We also attempted to synthesize dephosphocoenzyme A by heating these components of pantetheine with ADP and ATP. Heating for 24 h at 120 °C gave no detectable yield (<0.1%). This result is in accord with the known difficulty of synthesizing phosphodiester bonds under prebiotic conditions. The addition of ADP to pantetheine may instead have first occurred within living systems and may well need a ribozyme to catalyze the reaction.

These results suggest that pantetheine could have been a component of the prebiotic soup, and also that amide bond formation may have been possible on the primitive Earth at temperatures low enough not to destroy organic compounds. The requirement for these low-temperature condensations seems to be the presence of extremely concentrated aqueous solutions of reactants. □

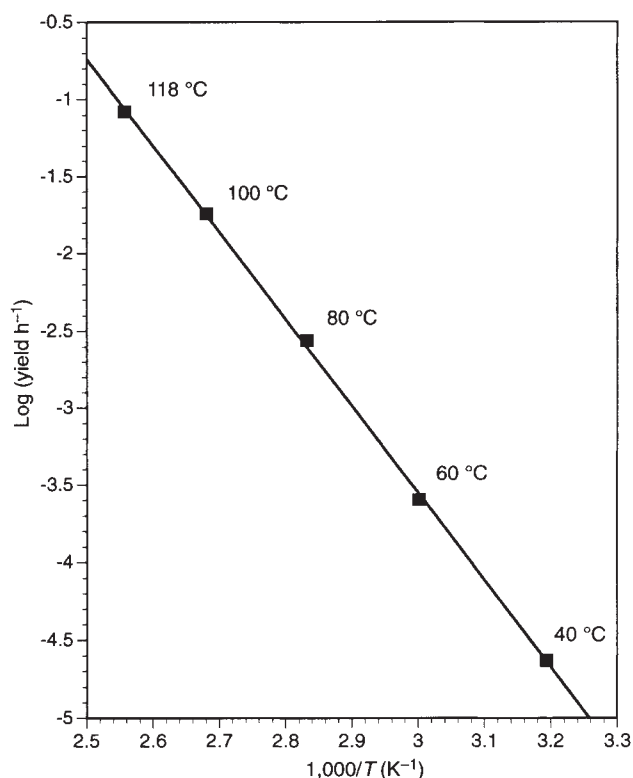


FIG. 4 Arrhenius plot of the initial rate of formation of pantetheine calculated from a linear plot of the first three points in Fig. 3 as a function of reciprocal temperature. The 40 °C point is from the yield of 0.018% after 31 days and 0.034% after 62 days.

Received 7 November 1994; accepted 17 January 1995.

1. Torchinsky, Y. M. *Sulfur in Proteins* (Pergamon, Oxford, 1981).
2. Lakin-Thomas, P. L. & Brody, S. *Eur. J. Biochem.* **146**, 141–147 (1985).
3. de Duve, C. *Blueprint for a Cell: The Nature and Origin of Life* (Patterson, Burlington, North Carolina, 1991).
4. Gesteland, R. F. & Atkins, J. A. (eds.) *The RNA World* (Cold Spring Harbor Laboratory Press, New York, 1993).
5. Schlesinger, G. & Miller, S. L. *J. Molec. Evol.* **36**, 302–307 (1993).
6. Schlesinger, G. & Miller, S. L. *J. Molec. Evol.* **36**, 308–314 (1993).
7. Fuller, W. D., Sanchez, R. A. & Orgel, L. E. *J. Molec. Biol.* **67**, 25–33 (1972).
8. Fuller, W. D., Sanchez, R. A. & Orgel, L. E. *J. Molec. Evol.* **1**, 249–257 (1972).
9. Miller, S. L. & Orgel, L. E. *The Origins of Life on The Earth* (Prentice Hall, Englewood Cliffs, NJ, 1974).
10. Miller, S. L. *Science* **117**, 528–529 (1953).
11. Kvenvolden, K. A., Lawless, J. G. & Ponnamperuma, C. *Proc. Natn. Acad. Sci. U.S.A.* **68**, 486–490 (1971).
12. Peltzer, E. T., Bada, J. L., Schlesinger, G. & Miller, S. L. *Adv. Space Res.* **4**, 69–74 (1984).
13. Gunning, H. E. & Strausz, O. P. *Adv. Photochem.* **4**(12), 143–194 (1966).
14. Fahey, R. C. & Newton, G. L. *Meth. Enzymol.* **143**, 85–96 (1987).
15. Bishop, M. J., Lohmann, R. & Orgel, L. E. *Nature* **237**, 162–164 (1972).
16. Lohmann, R. *J. Molec. Evol.* **10**, 137–154 (1977).
17. Fox, S. W. & Dose, K. *Molecular Evolution and the Origin of Life* (Freeman, San Francisco, 1972).
18. Rohlfing, D. L. *Science* **193**, 68–70 (1976).
19. Smith, E. R. B. & Smith, P. K. *J. biol. Chem.* **132**, 47–56 (1940).

ACKNOWLEDGEMENTS. We thank R. Fahey for assistance with the pantetheine analysis, and the NASA Specialized Center of Research and Training in Exobiology for a fellowship (A.D.K.) and grant support (S.L.M.).

Isotopic constraints on carbon exchange between deep ocean sediments and sea water

James E. Bauer*, Clare E. Reimers†, Ellen R. M. Druffel‡ & Peter M. Williams§

* School of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062-1346, USA

† Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, New Jersey 08903-0231, USA

‡ Department of Earth System Science, University of California, Irvine, California 92717, USA

§ Marine Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0128, USA

THE vast reservoirs of organic carbon in marine sediments^{1–3} have the potential to influence the properties of organic matter in the overlying water column. For example, it has been suggested that marine sediments are a possible source of the old, refractory dissolved organic carbon (DOC) found in deep water^{3,4}. Natural radiocarbon and stable carbon isotope ratios ($\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$) can be used to constrain the role of sediments in the ocean carbon cycle^{5–8}. Here we report the distributions of $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ associated with dissolved organic and inorganic carbon in sediment pore water, together with those of the particulate sedimentary organic carbon, from two geochemically distinct marine environments. Concentration gradients of dissolved organic and inorganic carbon across the sediment–water interface imply significant diffusive fluxes of these solutes from the sediment to the water column. But the DOC fraction in the sediments is greatly enriched in ^{14}C compared with that in the overlying sea water (by as much as 370‰), indicating that the DOC supplied by sediments to ocean waters must be relatively young, and that its remnant ages in the water column itself.

Sediments were collected from two regions of the eastern North Pacific Ocean. Station N (35° 00' N, 123° 00' W) is located in 4,100 m of water on the continental rise at the base of the Monterey deep sea fan⁹. Santa Monica basin (33° 45' N, 118° 45' W), a semi-enclosed, nearly anoxic basin in the California continental borderland, has an average mid-basin depth¹⁰ of ~900 m. The $\Delta^{14}\text{C}$ values of sedimentary organic carbon (SOC) were highly dissimilar between the two sites (Fig. 1a, b; Table 1). Relative to modern, pre-bomb, carbon in surface ocean waters ($\Delta^{14}\text{C} \approx -70\text{‰}$)¹¹, the SOC of surface sediments at station N was uniformly depleted in ^{14}C (average $\Delta^{14}\text{C} = -237 \pm 3\text{‰}$, $n = 6$, 0–1.5 cm). The $\Delta^{14}\text{C}$ values were markedly lower in deeper layers ($-344 \pm 7\text{‰}$ at 9.5 cm). In contrast to station N, the SOC from the central Santa Monica basin had a clear post-bomb ^{14}C signal ($\Delta^{14}\text{C} > -50\text{‰}$) to at least 2 cm depth. The steep $\Delta^{14}\text{C}$ gradient in SOC ($\Delta^{14}\text{C}$ -SOC) between ~3 cm depth and the sediment surface at Santa Monica is a result of the increasing global inventory of ^{14}C during atmospheric weapons testing of the 1950s and 1960s, while the much lower gradient from ~5–10 cm reflects ^{14}C decay over time.

Concentrations of DOC in sea water overlying the sediments (DOC_{olw}) of each site were low (~40 μM), and increased to $428 \pm 8 \mu\text{M}$ and $453 \pm 8 \mu\text{M}$ in surficial (0–0.25 cm depth) pore waters (DOC_{pw}) at station N and Santa Monica basin, respectively (Table 1). The DOC_{pw} increased to higher values in the basin (706 μM at 8.5 cm) than in station N (500 μM at 8.5 cm) sediments. This may be due to greater SOC content and anoxia in the former location, leading to greater production but less efficient respiration of DOC_{pw} .

There was close agreement between the $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ of SOC and DOC_{pw} in shallow station N sediments (Fig. 1a, Table 1). The simplest explanation for this is biotic and abiotic solubiliza-

tion of SOC to DOC. Particulate organic carbon (POC) sinking from the water column to the sediment surface is comparatively enriched in ^{14}C (–39 to +136‰)⁵. Thus the greater similarity of $\Delta^{14}\text{C}$ - DOC_{pw} to 'average' $\Delta^{14}\text{C}$ -SOC indicates that DOC_{pw} in station N surface sediments is derived from a mixture of young and old components comprising the bulk SOC rather than from exclusively young, biologically labile POC. The increasing difference between the $\Delta^{14}\text{C}$ of DOC_{pw} and SOC with sediment depth may result from long-term diagenetic reactions and differential diffusion of DOC_{pw} components within sediments.

At Santa Monica basin, all $\Delta^{14}\text{C}$ values of DOC_{pw} were lower than those of SOC (Fig. 1b, Table 1). The greatest difference (~260‰) between the $\Delta^{14}\text{C}$ of DOC_{pw} and SOC (similar to sinking POC⁵) was in the shallowest sediment layer (0–0.25 cm), suggesting that DOC_{olw} may diffuse into (or be mixed with) near-surface pore water. We calculate that if surficial pore waters contained DOC_{olw} with an assumed concentration of ~40 μM and $\Delta^{14}\text{C}$ of –462‰, the undiluted DOC_{pw} at 0–0.25 cm must have an average $\Delta^{14}\text{C}$ of ~–230‰, close to that observed, but significantly lower than the average bulk $\Delta^{14}\text{C}$ of SOC at this depth. The depletion in ^{14}C of DOC_{pw} in deep sediments of station N (Fig. 1a) and shallow sediments of Santa Monica basin (Fig. 1b) may also result from (1) age-fractionated diffusion of DOC_{olw} components into pore waters (the net flux of DOC is out of the sediments) or (2) selective dissolution and enzymatic hydrolysis of SOC fractions¹², coupled with the different pathways of respiration (that is, DOC-utilization) dominating at each site and depth.

The $\delta^{13}\text{C}$ values and concentrations of porewater dissolved inorganic carbon (DIC_{pw}) in marine sediments reflect contributions from DOC_{pw} and/or SOC mineralization, mixing with DIC from the overlying water column (DIC_{olw}), and CaCO_3 dissolution^{6,8,13,14}. The more rapid changes in DIC_{pw} concentrations and $\delta^{13}\text{C}$ values with depth at Santa Monica basin compared to station N indicate that a greater percentage of the porewater DIC at the former location is derived from isotopically light marine organic matter ($\delta^{13}\text{C} \approx -18$ to –22‰) than

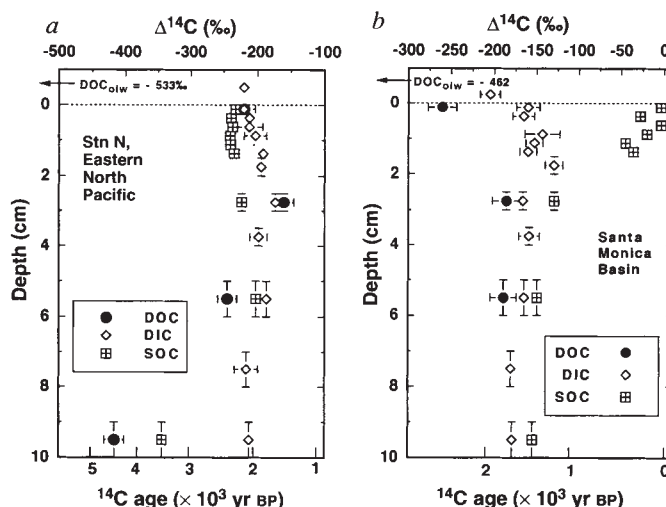


FIG. 1 Depth profiles of $\Delta^{14}\text{C}$ signatures of porewater DOC, DIC and solid-phase SOC in sediments from station N in the eastern North Pacific (a) and Santa Monica basin (b). Vertical bars are depth intervals for each data point. Horizontal bars are $\pm 1\sigma$ of $\Delta^{14}\text{C}$ measurement. Symbols without bars have depth intervals or σ values smaller than the symbols. DOC_{olw} indicates here $\Delta^{14}\text{C}$ values of DOC in sea water overlying sediments (off-scale relative to all other $\Delta^{14}\text{C}$ values at both sites). Dashed horizontal lines represent the sediment–water interface. See Table 1 legend for details of sample processing.

TABLE 1 Carbon concentrations and isotopic ratios of DOC, DIC and SOC in pore waters and sediments

Site	Depth interval (cm)	DOC			DIC			SOC	
		μM	$\delta^{13}\text{C}^*$ (‰)	$\Delta^{14}\text{C}^\dagger$ (‰)	μM	$\delta^{13}\text{C}^*$ (‰)	$\Delta^{14}\text{C}^\dagger$ (‰)	$\delta^{13}\text{C}^*$ (‰)	$\Delta^{14}\text{C}^\dagger$ (‰)
Stn N	OLW [‡]	40	-20.8	-533	2,376	0.0	-220		
	0-0.25	428§	-21.9	-220	2,432	-0.2	-220	-21.8	-232
	0.25-0.5				2,394	-0.1	-212		-239
	0.5-0.75	359			2,390	-0.1	-212		-237
	0.75-1.0	279			2,551	-0.6	-203		-241
	1.0-1.25	366			2,500	-0.6			-240
	1.25-1.5	753			2,516	-0.7	-191		-235
	1.5-2.0				2,671	-1.1	-194		
	2.0-2.5	474							
	2.5-3.0	500§	-20.8	-161	2,761	-1.5	-173	-21.8	-223
	3.0-3.5	559							
	3.5-4.0				2,769	-1.7	-198		
	4.0-5.0	442							
	5.0-6.0	434§	-22.1	-245	2,804	-1.8	-186	-21.8	-202
	6.0-7.0	463							
	7.0-8.0				2,742	-2.0	-217		
	8.0-9.0	495							
	9.0-10.0	508§	-22.9	-417	2,813	-2.0	-212	-21.9	-344
SMB	OLW [‡]	42	-20.8	-462	2,329	-0.1	-205		
	0-0.25	453							
	0-0.25	465§	-21.2	-260	2,468	-1.2	-160	-21.5	-4
	0.25-0.5	413			2,570	-2.4	-165		-28
	0.5-0.75	551							-4
	0.75-1.0	546			2,634	-2.4	-144		-20
	1.0-1.25	529			2,665	-2.7	-153		-45
	1.25-1.5	557			2,685	-2.9	-160		-36
	1.5-2.0				2,817	-3.5	-130		
	2.0-2.5	530							
	2.5-3.0	729§	-21.8	-186	3,133	-4.6	-166	-21.6	-130
	3.5-4.0				3,300	-5.4	-159		
	4.0-5.0	775							
	5.0-6.0	812§	-22.5	-189	3,516	-6.7	-165	-22.3	-150
	6.0-7.0	687							
	7.0-8.0				3,780	-7.8	-180		
	8.0-9.0	706							
	9.0-10.0				4,012	-8.8	-179	-22.2	-155

DOC, dissolved organic carbon; DIC, dissolved inorganic carbon; SOC, sedimentary organic carbon. Surface ocean waters at station N (Stn N) have a summer maximum in primary productivity derived from upwelled nutrients, a fraction of which is exported episodically to the sea floor leading to an increase in surface sediment organic-matter content from June to August^{20,21}. A ²¹⁰Pb profile, bottom photography and core X-radiography also demonstrate^{9,21,22} that these sediments are highly bioturbated to 5–6 cm depth. Sediments at Santa Monica basin (SMB) have a surface microbial mat that largely consists of iron-oxidizing bacteria; the sediments are laminated, indicating minimal disturbance by bioturbating infauna¹⁰. Oxygen is depleted by ~3 cm depth at station N and 2–3 mm depth at SMB²². The limit of detectable nitrate penetration at both sites is ~3 cm depth^{9,23}. Total sediment accumulation rates have been estimated to be 12 mg cm⁻² yr⁻¹ at station N²⁴ and 14–24 mg cm⁻² yr⁻¹ at SMB^{25,26}.

METHODS. Subcores from Soutar box cores⁹ or gravity cores were sectioned at 0.25-cm intervals over the top 1.5 cm, 0.5-cm intervals from 1.5 to 6 cm, and 1.0-cm intervals at greater depth. All sample processing was performed close to *in situ* temperature under oxygen-free conditions. The potential effects of pressure and temperature changes on sediment organic matter during sample retrieval have been noted, but not evaluated, in previous studies²⁷ because of limitations in sampling technology. Pore water was separated from bulk sediments by centrifugation at 5,000 r.p.m., a speed which was not found to induce DOC_{pw} sampling artefacts²⁷, and filtered through pre-combusted glass fibre filters. Subsamples for DOC_{pw} and SOC analyses were immediately frozen at -20 °C. Pore water for DIC analyses was treated with a saturated HgCl₂ solution and refrigerated at 4 °C. Detailed profiles of DOC_{pw} concentrations were determined by discrete-injection (50 µl) high-temperature catalytic oxidation (HTCO)²⁸. For $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ analyses of DOC_{pw}, 10–20 ml samples were acidified to pH 2–2.5 with 10% H₃PO₄, sparged free of inorganic carbon, and oxidized to CO₂ by continuous-injection HTCO^{5,15}. The DOC_{pw} was oxidized by irradiation of 650-ml samples with a 2,400-W ultraviolet light source, and procedural $\Delta^{14}\text{C}$ -DOC blanks were determined periodically^{5,15}. Samples (5–10 ml) for DIC analyses were acidified to pH ~2, and total CO₂ was sparged and collected⁸. SOC samples were acidified for 24 h with 3% H₃PO₄ to remove carbonates, dried, and combusted to CO₂ in sealed quartz tubes at 850 °C (ref. 29). Dry-weight sediment organic and carbonate carbon contents in the uppermost 10 cm were, respectively: station N, 1.5–2% and 0.1–0.2%; SMB, 4.5–5% and 1.3–2.3%; J.E.B., unpublished data). Average measurement errors ($\pm 1\sigma$) for each analysis are: DOC, 8 µM; DIC, ± 1 µM; $\delta^{13}\text{C}$, ± 0.2 ‰; $\Delta^{14}\text{C}$ -DOC, ± 15 ‰; $\Delta^{14}\text{C}$ -DIC, ± 12 ‰; $\Delta^{14}\text{C}$ -SOC, ± 7 ‰.

* $\delta^{13}\text{C} = ([R_{\text{sample}}/R_{\text{standard}}] - 1) \times 1,000$, where $R = {}^{13}\text{C}/{}^{12}\text{C}$ as measured by isotope-ratio mass spectrometry.

† $\Delta^{14}\text{C}$ is defined as the deviation in parts per thousand from the ¹⁴C activity of nineteenth-century wood and is calculated in the conventional manner³⁰. All $\Delta^{14}\text{C}$ analyses were made at the Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory, on total amounts ranging from ≤ 100 µg (DOC) to ~1,000 µg (DIC, SOC) of carbon following the conversion of sample CO₂ to graphite targets³¹.

‡ OLW, values for DOC and DIC in sea water overlying sediments at each site.

§ DOC_{pw} concentrations determined by continuous-injection HTCO during processing of $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ samples. All reported DOC concentrations and $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ values were corrected by the corresponding system blanks (~3–5 µM C for discrete injection; 0.2 µmol C for continuous injection^{5,15,28}).

TABLE 2 Calculated and measured fluxes of DOC and DIC

Site	Calculated fluxes		Measured fluxes	Source of measured flux
	DOC ($\text{g C m}^{-2} \text{ yr}^{-1}$)	DIC	DIC ($\text{g C m}^{-2} \text{ yr}^{-1}$)	
Stn N	12.8	6.3	8.3 ± 2.3	Ref. 9
SMB	16.5	19.0	11.8 ± 5.8	Ref. 10
Average	14.7 ± 2.6	12.7 ± 9.0		

Calculated fluxes assume a fickian diffusion model⁹ of $F_s = \phi D_{\text{sed}}(\Delta C_s/\Delta z)$, where F_s is the flux of the solute of interest, ϕ is sediment porosity (0.90 and 0.96 cm^3 pore water cm^{-3} sediment at 0–0.25 cm for station N and SMB, respectively), D_{sed} is the sediment diffusion coefficient for each solute and is equal to $D_{\text{sw}}\phi^2$ (where D_{sw} , the diffusion coefficient in sea water, is conservatively estimated to be $1.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for $\text{DOC}^{27,32}$ and $5.1 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for HCO_3^- (refs 13, 33) at both sites, irrespective of slight differences in bottom-water temperature of 1.5 and 5 °C for station N and SMB, respectively), ΔC_s is the difference in concentration of each solute between the shallowest sediment layer and the overlying sea water, and Δz is the distance of the mid-point of the shallowest sediment layer from the sediment–water interface (0.125 cm). For calculated flux estimates, it was assumed that DOC_{pw} concentrations were not affected by sampling artefacts due to pressure and temperature changes during core retrieval or by methodological constraints limiting the sectioning interval of sediments to 0.25 cm. Measured fluxes were determined *in situ* using benthic flux chambers^{9,10}.

from CaCO_3 ($\delta^{13}\text{C} = \sim 0$ to $+1\%$) dissolution (Table 1, Fig. 2). From pH profiles measured *in situ*¹⁰ and our DIC_{pw} profiles from both sites (Table 1), we have estimated that pore waters at Santa Monica basin reach saturation ($\Omega = 1.0$) with respect to calcite within 0.75 cm of the sediment–water interface and that significant CaCO_3 dissolution does not occur, similar to previously reported findings for the central basin¹⁰. These findings lead us to conclude that, to a first approximation, the change in $\delta^{13}\text{C}$ of DIC_{pw} as a function of DIC_{pw} concentration in sediments in

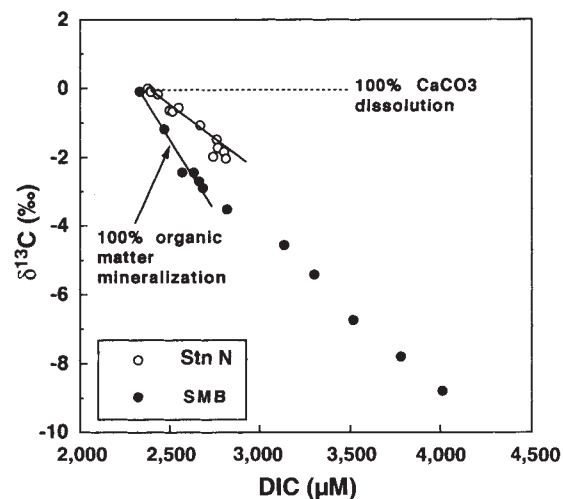


FIG. 2 Plot of $\delta^{13}\text{C}$ values of DIC versus concentration of DIC in pore water from station N and SMB. Regression of SMB $\delta^{13}\text{C}$ values as a function of DIC concentration from 0 to 1.5 cm depth ($n=6$) yields $\Delta\delta^{13}\text{C}/[\text{DIC}] = -0.00787$, $r^2 = 0.9694$. For station N, $\Delta\delta^{13}\text{C}/[\text{DIC}] = -0.00370$ from 0 to 3 cm depth ($n=9$), $r^2 = 0.9704$. It is assumed that SMB pore waters are saturated at all depths with respect to calcium carbonate, and that the change of $\delta^{13}\text{C}$ and DIC is due entirely to organic matter mineralization. The $\delta^{13}\text{C}$ of DIC at station N is calculated as being derived from an admixture of DIC from organic-matter mineralization (represented by the SMB regression) and CaCO_3 (0‰ with respect to $\delta^{13}\text{C}$) dissolution. The fraction of station N DIC derived from organic-matter mineralization is given by $F_{\text{OM}}(0.00787) + (1 - F_{\text{OM}}) = 0.0037$, where $F_{\text{OM}} = 0.47$.

Santa Monica basin can be attributed to organic-matter degradation alone. In contrast, station N pore waters are highly undersaturated ($\Omega = 0.5\text{--}0.8$) to at least 8 cm depth. A regression of $\delta^{13}\text{C}$ of DIC_{pw} as a function of DIC_{pw} concentration for both sites (Fig. 2) allows the fraction of DIC production from organic-matter degradation at station N to be calculated. Our estimate of 47% agrees closely with an independent estimate of $52 \pm 7\%$ made by comparing benthic fluxes of DIC to total organic-carbon degradation rates⁹.

The $\Delta^{14}\text{C}$ values for DIC_{pw} at Santa Monica basin are in all cases intermediate between the $\Delta^{14}\text{C}$ of SOC and DOC_{pw} , or SOC and DIC_{olw} (Fig. 1b). Concentrations and $\Delta^{14}\text{C}$ values of DIC_{pw} in excess of DIC_{olw} concentrations and $\Delta^{14}\text{C}$ thus reflect inputs resulting from respiration of DOC_{pw} and SOC at each relevant depth horizon. Assuming that the major sources of DIC_{pw} are known and that to a first approximation bioturbation is negligible at this site, a mass balance for the $\Delta^{14}\text{C}$ of DIC_{pw} is

$$\Delta^{14}\text{C-DIC}_{\text{pw}} = ([\text{DIC}_{\text{olw}}]/[\text{DIC}_{\text{pw}}])\Delta^{14}\text{C-DIC}_{\text{olw}} + (([\text{DIC}_{\text{pw}}] - [\text{DIC}_{\text{olw}}])/[\text{DIC}_{\text{pw}}])\Delta^{14}\text{C}_{\text{source}} \quad (1)$$

where subscript pw indicates pore water, subscript olw indicates sea water overlying sediments (also equivalent to pore water initially sequestered owing to sediment burial), $([\text{DIC}_{\text{pw}}] - [\text{DIC}_{\text{olw}}])$ is the DIC added to pore waters due to the respiration of organic carbon, and $\Delta^{14}\text{C}_{\text{source}}$ is its radiocarbon signature. Using equation (1), we calculate that the source material of the DIC added to pore water has an average $\Delta^{14}\text{C}$ value of -55% at 2.5–3 cm depth and -85% at 5–6 cm depth. These values are more similar to ^{14}C -enriched SOC than ^{14}C -depleted DOC_{pw} at Santa Monica basin (Table 1). In addition, selective mineralization of ^{14}C -enriched components of either the bulk DOC or SOC may further result in the addition of ^{14}C -enriched DIC to pore water.

A similar evaluation of the relative contribution of DOC_{pw} and SOC to the $\Delta^{14}\text{C}$ values of DIC_{pw} at station N is not possible at present because of uncertainties in the $\Delta^{14}\text{C}$ of the biogenic calcite raining to the sea floor. However, increasing divergence in the $\Delta^{14}\text{C}$ of DIC_{pw} relative to the DOC_{pw} and SOC pools with depth (that is, 10 cm; Fig. 1a) indicates that (1) DIC_{pw} is unaffected by large changes in $\Delta^{14}\text{C}$ of SOC and DOC_{pw} due to little net DIC addition below 3 cm (Table 1) or (2) selective mineralization of small amounts of 'young', downwardly mixed and isotopically distinct fractions of SOC and/or DOC_{pw} may occur within the mixed layer of the sediment column.

The conspicuous concentration gradients of DOC and DIC across the sediment–water interface at both sites suggest that there are significant fluxes of these isotopically distinct solutes to the water column. Using a simplified fickian diffusion model (Table 2), we calculate fluxes (combined averages of both sites) to the overlying water column of $15 \pm 3 \text{ g C m}^{-2} \text{ yr}^{-1}$ for DOC and $13 \pm 9 \text{ g C m}^{-2} \text{ yr}^{-1}$ for DIC. The calculated DIC fluxes for both sites agree within measurement error with previously determined fluxes measured *in situ* using benthic flux chambers^{9,10} (Table 2). Thus, reduced carbon in the form of DOC may be released back to the water column in amounts equal to fully mineralized carbon, representing an export of both carbon and energy from these sediments.

Porewater DOC at both sites was greatly enriched in ^{14}C relative to DOC_{olw} (Fig. 1a, b; Table 1). The $\Delta^{14}\text{C}$ values of both DOC pools are expected to be more similar if pore water was a major source of DOC residing over the long term within the deep-water column, but elevated $\Delta^{14}\text{C}$ values of deep-ocean DOC have not been found^{4,5,15}. We interpret these observations to indicate either that ^{14}C -enriched DOC_{pw} is not released from sediments in significant quantities or that it does not persist in the water column. One way that fluxes of DOC could potentially be overestimated by up to 1–3 orders of magnitude is by the microbial utilization of labile DOC at the oxygen- and nutrient-

rich sediment–water interface¹⁶ (that is, narrower than the top 0–0.25 cm sampling interval); this would lead to a cross-interface DOC gradient that is smaller than that implied by our measurements (Table 1). Both the calculated and measured fluxes (Table 2) indicate that oxidation of DOC_{pw} at the interface could potentially account for much of the DIC flux. Rapid utilization of DOC_{pw} by water-column bacteria following its exchange would also diminish the presence of DOC_{pw} in the water column.

Using average upper water column mixed-layer DOC concentrations of 60 μM and deep-water concentrations of 40 μM (refs 4, 5), the inventory of DOC from 0–4,000 m in an area such as station N is 2,200 g C m^{-2} . If DOC_{pw} persists in the water column, our upper estimate of sediment–water DOC flux of 15 $\text{g C m}^{-2} \text{ yr}^{-1}$ leads to a lower estimated replacement time of ≥ 150 yr. Lower flux rates would lead to greater replacement times. Current and previous estimates^{4,15,16} of the average apparent ¹⁴C age of water-column DOC below ~1,000 m depth in different regions of the North Pacific are ~6,000 yr. Although pore water may be a significant source of DOC to sea water, our findings indicate that sediments are unlikely to provide pre-aged DOC to the oceans, thus limiting the strength of this source over the long term (10^3 – 10^4 yr). Instead, the DOC supplied by sediments to ocean waters must be relatively young and, in a manner similar to its ¹⁴C-enriched riverine counterpart^{3,17–19}, ages and undergoes degradation within the ocean itself. □

Received 24 August 1994; accepted 13 January 1995.

1. Emerson, S. & Hedges, J. I. *Paleoceanography* **3**, 621–634 (1988).
2. Berner, R. A. & Canfield, D. E. *Am. J. Sci.* **289**, 333–361 (1989).

3. Hedges, J. I. *Mar. Chem.* **39**, 67–93 (1992).
4. Williams, P. M. & Druffel, E. R. M. *Nature* **330**, 246–248 (1987).
5. Druffel, E. R. M., Williams, P. M., Bauer, J. E. & Ertel, J. R. *J. geophys. Res.* **97**, 15639–15659 (1992).
6. Bauer, J. E., Haddad, R. I. & Des Marais, D. J. *Mar. Chem.* **33**, 335–351 (1991).
7. Bauer, J. E., Spies, R. B., Vogel, J. S., Nelson, D. E. & Southon, J. R. *Nature* **348**, 230–232 (1990).
8. McCorkle, D. C. & Emerson, S. R. *Geochim. cosmochim. Acta* **52**, 1169–1178 (1988).
9. Reimers, C. E., Jahnke, R. A. & McCorkle, D. C. *Glob. biogeochem. Cycles* **6**, 199–224 (1992).
10. Jahnke, R. A. *J. mar. Res.* **48**, 413–436 (1990).
11. Berger, R., Taylor, I. E. & Libby, W. F. *Science* **153**, 864–866 (1966).
12. Trumbore, S. E., Schiff, S. L., Aravena, R. & Elgord, R. *Radiocarbon* **34**, 626–635 (1992).
13. McCorkle, D. C., Emerson, S. R. & Quay, P. D. *Earth planet. Sci. Lett.* **74**, 13–26 (1985).
14. McNichol, A. P., Lee, C. & Druffel, E. R. M. *Geochim. cosmochim. Acta* **52**, 1531–1543 (1988).
15. Bauer, J. E., Williams, P. M. & Druffel, E. R. M. *Nature* **357**, 667–670 (1992).
16. Jahnke, R. A., Craven, D. B. & Gaillard, J.-F. *Geochim. cosmochim. Acta* **58**, 2799–2809 (1994).
17. Maybeck, M. *Am. J. Sci.* **282**, 401–450 (1982).
18. Meyers-Schulte, K. J. & Hedges, J. I. *Nature* **321**, 61–63 (1986).
19. Hedges, J. I. et al. *Science* **231**, 1129–1131 (1986).
20. Smith, K. L., Baldwin, R. J. & Williams, P. M. *Nature* **359**, 313–316 (1992).
21. Smith, K. L., Kaufman, R. S. & Baldwin, R. J. *Limnol. Oceanogr.* **39**, 1101–1118 (1994).
22. Cai, W.-J. Thesis, Univ. California (1992).
23. Shaw, T. et al. *Geochim. cosmochim. Acta* **54**, 1233–1246 (1990).
24. Cai, W.-J., Reimers, C. & Shaw, T. *Geochim. cosmochim. Acta* (in the press).
25. Bruland, K., Bertine, K., Koide, M. & Goldberg, E. D. *Envir. Sci. Technol.* **8**, 425–432 (1974).
26. Finney, B. P. & Huh, C.-A. *Envir. Sci. Technol.* **23**, 294–303 (1989).
27. Martin, W. R. & McCorkle, D. C. *Limnol. Oceanogr.* **38**, 1464–1479 (1993).
28. Williams, P. M., Bauer, J. E., Robertson, K. J., Wolgast, D. M. & Ocelli, M. L. *Mar. Chem.* **41**, 271–281 (1993).
29. Sofer, Z. *Analyt. Chem.* **52**, 1389–1391 (1980).
30. Stuiver, M. & Polach, H. A. *Radiocarbon* **19**, 355–363 (1977).
31. Vogel, J. S., Nelson, D. E. & Southon, J. R. *Radiocarbon* **29**, 323–333 (1987).
32. Burdige, D. J., Alperin, M. J., Homstead, J. & Martens, C. S. *Geophys. Res. Lett.* **19**, 1851–1854 (1992).
33. Li, Y.-H. & Gregory, S. G. *Geochim. cosmochim. Acta* **20**, 131–140 (1974).

ACKNOWLEDGEMENTS. We thank J. Southon, S. Trumbore and M. Kashgarian for assistance with $\Delta^{14}\text{C}$ analyses; S. Griffin and D. Wolgast for help with sample preparation; J. Hedges, J. Chanton and W. Martin for comments and suggestions; and the crew of the RV *New Horizon* for help with sample collection and ship logistics. This work was supported by the US NSF.

Explosive fragmentation of erupting magma

Ichiro Sugloka*† & Marcus Bursik†

* Graduate Aeronautics Laboratory, California Institute of Technology, Pasadena, California 91125, USA

† Department of Geology, State University of New York at Buffalo, Buffalo, New York 14260, USA

THE magma responsible for explosive volcanic eruptions has both a volatile and an inert phase. Deep in the conduit of an active volcano, bubbles nucleate as the volatiles exsolve^{1–3}. As the magma rises, the bubbles grow through depressurization and continued exsolution. It is thought that when the pressure in the bubbles exceeds that in the overlying material, the magma undergoes a rapid transformation from a continuous magmatic phase with bubbles to a continuous gas phase with fragmented pyroclastic material^{1,2}. The fragmentation process is complex and poorly understood. To understand better how the transport of fragmented material is coupled to exsolution and vaporization, we have performed depressurization experiments on a two-phase system, designed to simulate the eruption process. We identify a new explosive vaporization process, in which a fragmentation front propagates downwards through a mixture of volatile liquid and inert particulate material, suppressing the growth of nucleated bubbles by compressing the material ahead of it. This process is distinct from, and may complement, previously identified fragmentation mechanisms such as non-nucleate vaporization⁴ and fragmentation induced by an expanding magmatic foam⁵.

Because of the violence of the explosive process, conditions in an active volcanic conduit are not amenable to direct observation. In addition, the thermodynamics and fluid dynamics con-

trolling the flux of mass, momentum and energy are closely coupled and nonlinear². In magmatic eruptions, the volatile phase consists of water, CO₂ and other less-abundant dissolved components⁶, whereas the inert phase consists of a viscous silicate liquid, crystals and country rock. In the experiments (Fig. 1), vapour was rapidly produced by the sudden depressurization of a volatile liquid within a matrix of (inert) spherical particles. The two phases are analogous to the separate volatile and inert phases in erupting magma. Because the phases were intermingled, they were most like mechanically mixed magma and volatiles, as may occur in hydrovolcanic⁷ or magmatic⁸ systems. The particles not only served as the material to be fragmented and transported, but also provided bubble nucleation sites virtually identical to the crystal surfaces that perform this role in natural melts³. Both settings involve the nucleation and growth of bubbles by depressurization and diffusion, and the subsequent transport of material by the released vapour. The process of vaporization in the experiments is an analogue for exsolution and vaporization in explosive eruptions. Depressurization below the vapour pressure of a volatile liquid is equivalent to instantaneously and uniformly superheating it⁴, and is analogous to supersaturating a dissolved phase. The experiments were performed with different volatile components and different dump-tank pressures to simulate differences in saturation vapour pressure, and hence volatile content in magma. A centrifuge was used to simulate magma columns up to 50 m deep, and quasi-steady eruption conditions lasting for several seconds^{9,10}. By transformation into the reference frame of a rising magma, steady phenomena were probably simulated.

The vaporization of the volatile liquid is a thermodynamic process that converts the internal energy of the fluid into the kinetic energy of fragmentation and expulsion. The change in energy, ΔI , is initiated by depressurization at constant temperature, ΔP , and is given by $\Delta I = v\Delta P$ where $\Delta P = P_v - P_d$, the volatile vapour pressure minus the ambient, dump-tank (back)

† Present address: Volvo Monitoring and Concept Center, 700 Via Alondra, Camarillo, California 93012, USA.

pressure, and v is specific volume. Thus the ratio of the amount of energy used in an eruption to the total available is $\Delta P/P_v$. Because the vapour pressure is the analogue of the saturation vapour pressure, P_s , in magma¹, and the back pressure in nature is always atmospheric pressure, P_a , we have for a volcanic eruption

$$\frac{\Delta P}{P_v} \equiv \frac{P_s - P_a}{P_s} = 1 - \frac{P_a}{P_s}$$

Thus as the volatile content of magma increases, $\Delta P/P_v \rightarrow 1$ in our analogue experiments.

The vaporization (or exsolution) and eruption process can be viewed as an expansion of the erupting mixture against gravity. The most explosive events will be those exhibiting the greatest expansion of the mixture on fragmentation. Because expanded mixture that has left the test cell is deposited in the dump tank, the bulk density of the expanded particle dispersion just before fall-back (Fig. 1c) is proportional to h_2/h_0 (the depth of the particle layer in the test cell at the end of an experiment, h_2 ,

divided by the test cell length, h_0). Gravity restrains the vertical expansion process, so that the degree of expansion, h_2/h_0 , is decreased (or effective h_0 is increased) by applying centrifugal acceleration in the expansion direction. Because of the complicated nature of the multiphase flow within the test cell, a power-law relationship between centrifugal acceleration and h_2/h_0 was determined by experiment (Fig. 2a). The ratio h_2/h_0 was found to vary as $\sqrt{N}$, where N is centrifugal acceleration divided by gravitational acceleration.

The above arguments lead to the hypothesis that the relationship between depressurization and expansion takes the form:

$$h_2/\sqrt{N}h_0 = f(\Delta P/P_v)$$

In fact, as volatile vapour pressure, back pressure and centrifugal acceleration were varied, it was found that the amount of particles left in the test cell after each experiment did undergo systematic variations (Fig. 2b). A distinct change in $h_2/\sqrt{N}h_0$ over a narrow range of $\Delta P/P_v$ indicated the existence of two distinct expulsion processes, type 1 and type 2, and that the one that occurred was determined by the relative internal energy release.

When the test cell was vented at $\Delta P/P_v < R_c$, where the critical energy ratio, R_c , was ~ 0.45 in the experiments, a type 2 expulsion process occurred. Type 2 processes were characterized by simple nucleate boiling. Bubbles of vaporized volatile liquid grew

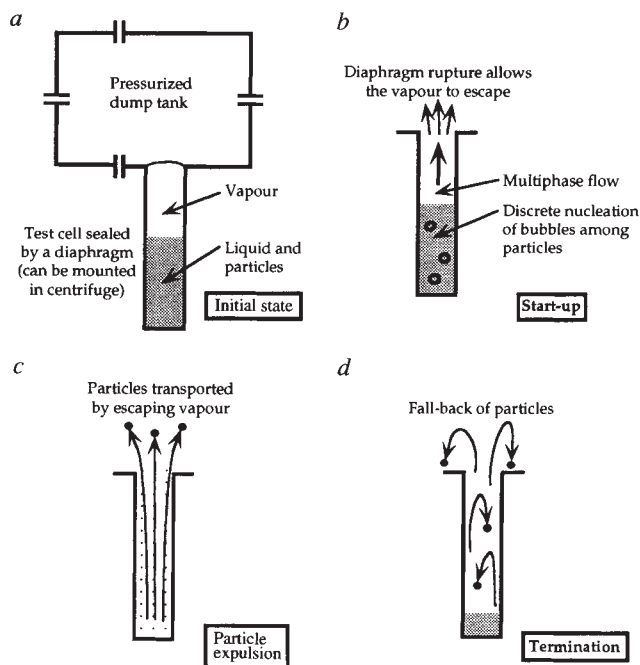


FIG. 1 In an experiment, the vaporization-driven transport process proceeds through four stages. In the initial state (a), a loose-packed layer of particles is immersed in a volatile liquid inside the test cell, pressurized to slightly above the liquid vapour pressure. During start-up (b), bubbles nucleate as test-cell pressure drops below vapour pressure. In the expulsion phase (c), the superheated liquid vaporizes, disrupting the particle layer and expelling particles against gravity. Termination (d) occurs when no superheated liquid remains.

METHODS. The test cell, a cylindrical pressure vessel consisting of a Pyrex pipe section (internal diameter 2.5 cm), vents upwards into a large, pressure-controlled ($P_d = 0\text{--}3.1$ bar) dump tank. The same cell was used^{9,10} in a centrifuge at accelerations up to $175g$. Two refrigerants acted as the volatile phase, each with a room-temperature vapour pressure, P_v , between 1 bar and the test-cell design limit— CCl_2F_2 (R12, $P_v = 6.5\text{--}8$ bar) and $\text{C}_2\text{Cl}_2\text{F}_4$ (R114, $P_v = 2\text{--}2.8$ bar). These liquids were kept below the superheat limit so that no homogeneous (non-nucleate) boiling could occur. Most experiments were conducted using monodisperse glass spherules, of which 99.5 wt% were $0.425\text{--}0.589$ mm in diameter. The particles were large enough to be individually resolved in 16-mm high-speed motion pictures, but being only 2% of the test cell in diameter, their movement was negligibly affected by the smooth test-cell walls. The particles (specific weight 2.95) remained packed at the bottom when the test cell was filled with liquid (specific gravity 1.3–1.45).

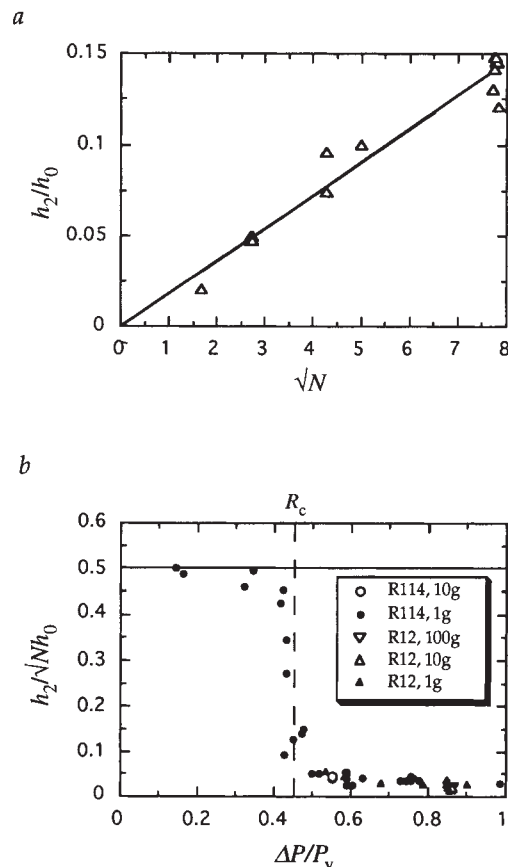


FIG. 2 a, Empirical determination of the relationship between centrifugal acceleration and bulk density of the particle dispersion at full expansion. Particles of diameter 0.5 mm in R12 with $P_v \approx 7$ bar were vented to $P_d = 1$ bar at a variety of centrifugal accelerations. The depth of the particle layer in the test cell following type 1 events (see text) was not dependent on the initial amount, but was instead $\propto \sqrt{N}$, where N is the centrifugal acceleration divided by the acceleration due to gravity, g . The depth measurements were accurate to <1 mm. b, The expansion, $h_2/\sqrt{N}h_0$, is a function of the relative energy release, $\Delta P/P_v$. The horizontal line indicates the initial depth of the mixture layer at $1g$. h_2 is the depth of the particle layer after an experiment; h_0 is the test-cell length.

rapidly throughout the test cell, leading to the large-scale breakup of the particle/volatile matrix. Bubbles at all depths expanded simultaneously to form vapour cavities throughout the matrix, hence particles at different depths were displaced simultaneously, as shown by the particle velocity data (Fig. 3a). During the expulsion phase, photographs revealed large clumps of wet particles (resulting from the large-scale breakup), held together by the surface tension of unvaporized volatile. Oscillations in the pressure at the base of the test cell, at a frequency consistent with the test cell acting as an acoustic cavity, were observed in some experiments directly after diaphragm rupture (Fig. 4a). In the example given, acoustic oscillations in the base pressure indicated the formation of vapour cavities extending the length of the test cell 10 ms after start-up. The low particle-expulsion yield in type 2 processes was probably a consequence of vapour escaping through these cavities, which were relatively free of particles.

In the type 1 particle expulsion process, at $\Delta P/P_v > R_c$, high-speed photographs showed explosive vaporization disrupting the particles within a narrow zone, a 'disruption front' which initiated at the top of the matrix and propagated through it, apparently with a self-similar form. The initial drop in pressure

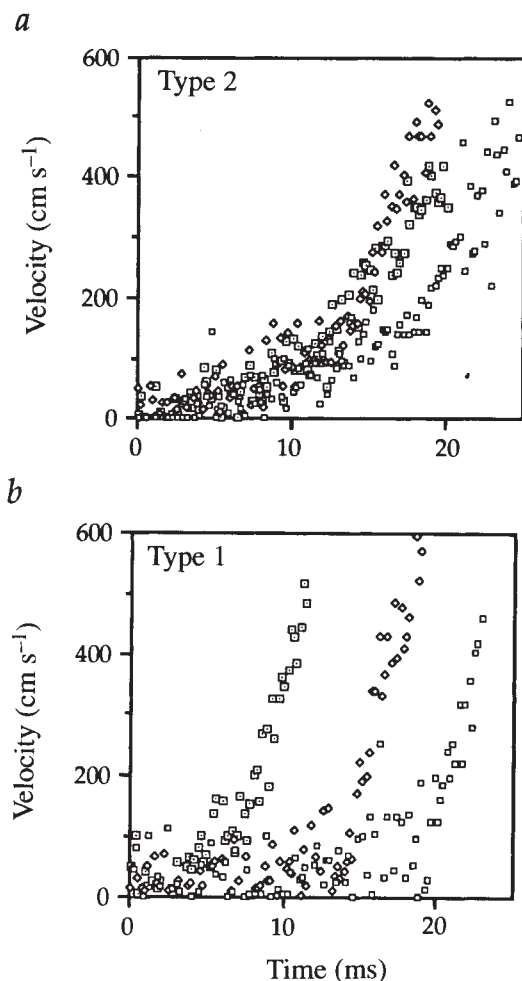


FIG. 3 a, The velocities of coloured particles in type 2 experimental eruptions demonstrate that particles throughout the length of the test cell are accelerated nearly simultaneously soon after eruption initiation. Test fluid is R114, $\Delta P/P_v = 0.43$, $h_2/\sqrt{Nh_0} = 0.093$. b, Velocities of labelled coloured particles expelled in a type 1 experimental eruption demonstrate that the particles are moved only as the disruption front passes, and that a discontinuity in acceleration exists. Test fluid is R12, $\Delta P/P_v = 0.862$, $h_2/\sqrt{Nh_0} = 0.019$. In a and b, individual symbols are for particles at different depths in the test cell; particles are separated by several centimetres vertically. a and b show the results of two separate experiments.

following diaphragm rupture (Fig. 4b) caused nucleation throughout the length of the test cell in experiments at $N=1$, and nucleation within the upper fraction of the test cell at $N \gg 1$. The hydrostatic pressure remained sufficiently high to inhibit bubble nucleation below a certain depth. In contrast to experiments exhibiting the type 2 process, no simultaneous particle displacement caused by nucleate boiling was observed (Fig. 3b), and bubbles remained minute until obscured by the front; their growth was apparently suppressed by compression of the matrix resulting from material being accelerated by the front (Fig. 4b). In the illustrated example, coloured particles were tracked as they were dispersed sequentially, each in a fashion similar to those above (Fig. 3b), and their sudden acceleration corresponds to the arrival of the front. The discontinuity in acceleration suggests that the disruption front is similar to a shock in a dense dusty gas¹¹. However, unlike a shock wave, the front is a rarefaction wave that expands and accelerates the particles behind it.

The disruption fronts propagated at 2–4 m s⁻¹ relative to the matrix. Although initiated by the rarefaction wave¹² that superheated the volatile material (speed > 500 m s⁻¹), they were much slower than this wave and not coupled to it; but they were still too fast to be studied in detail. However, high-speed photography did show a concentrated region of vaporization propagating through a medium that should support only pervasive nucleate vapour production. Thus although the details remain unclear, our results demonstrate the existence of an important new process for vaporization and fragmentation in the presence of abundant nucleation sites.

Because both the experimental and natural explosive eruptions involve the nucleation of bubbles of a superheated or supersatu-

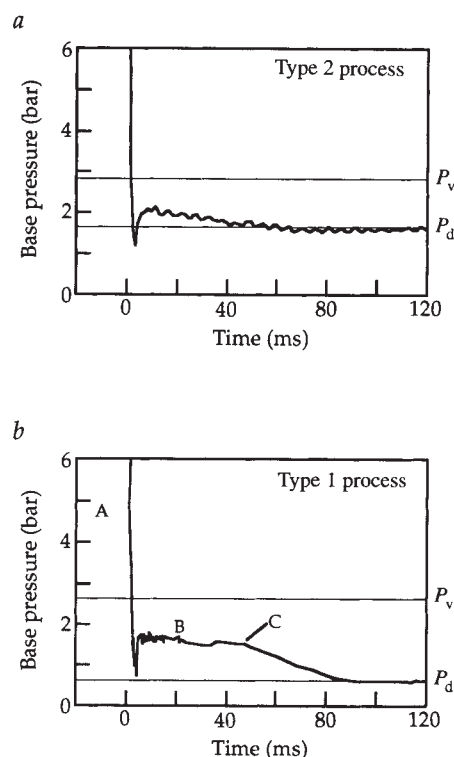


FIG. 4 a, The base-pressure traces of type 2 experimental eruptions, especially those for which $\Delta P/P_v \approx R_c$, display reverberations that are consistent with an open cavity extending the length of the test cell. Test fluid is R114, $\Delta P/P_v = 0.43$, $h_2/\sqrt{Nh_0} = 0.093$. b, Typical base-pressure trace for a type 1 event shows initial state (A), diaphragm rupture at time $t=0$, rarefaction and pressurization to P_d , compression during expulsion (plateau at B), followed by a monotonic decrease in base-pressure to dump-tank pressure caused by the arrival of the fragmentation front at the base of the test cell at C. Test fluid is R114, $\Delta P/P_v = 0.78$, $h_2/\sqrt{Nh_0} = 0.037$.

rated volatile phase within an inert matrix, we hypothesize that in natural eruptions, when $1 - P_a/P_s > R_c$, the type 1 process occurs. Because of the differences in detail between supersaturation and superheating, the value for R_c in natural eruptions, hence the critical volatile content, is not known. But the results do mean that, even in a magma with a high concentration of efficient nucleation sites³, it is possible that disruption and fragmentation are not always associated with (nor a function of) bubble growth, as is generally assumed^{1,2}. As the experimental disruption front clearly does not involve bubble growth, it may be that magma does not fragment at a critical bubble size and internal overpressure or magma viscosity^{1,13}. Rather, fragmentation may result from spontaneous exsolution and vaporization at an interface. □

Received 2 September 1994; accepted 10 January 1995.

1. Sparks, R. S. J. *J. Volcan. geotherm. Res.* **3**, 1–37 (1978).
2. Wilson, L., Sparks, R. S. J. & Walker, G. P. L. *Geophys. J. R. astr. Soc.* **63**, 117–148 (1980).
3. Hurwitz, S. & Navon, O. *Earth planet. Sci. Lett.* **122**, 267–280 (1994).
4. Hill, L. G. & Sturtevant, B. in *Adiabatic Waves in Liquid-Vapor Systems* 25–37 (Springer, New York, 1990).
5. Mader, H. M. et al. *Nature* **372**, 85–88 (1994).
6. Anderson, A. T. et al. *Geology* **17**, 221–225 (1989).
7. Sheridan, M. F. & Wohletz, K. H. *J. Volcan. geotherm. Res.* **17**, 1–29 (1983).
8. Gerlach, T. M. *Eos* **74**, 104–105 (1993) (Abstr.).
9. Ortiz, L. A., Scott, R. & Lee, J. *Int. J. Earthquake Engng Struct. Dynam.* **11**, 251–268 (1983).
10. Scott, R. *Int. J. Soil Dyn. Earthquake Engng* **2**, 188–198 (1983).
11. Marble, F. E. *Combustion and Propulsion: High Temperature Phenomena* (eds Hagerty, R. P. et al.) 175–213 (5th Agardograph Colloq., Macmillan, New York, 1963).
12. Wohletz, K., McGetchin, T. R., Sandford, M. T. & Jones, E. M. *J. Geophys. Res.* **89**, 8269–8286 (1984).
13. McBirney, A. R. & Murase, T. *Bull. volcan.* **34**, 372–384 (1970).

ACKNOWLEDGEMENTS. We thank A. Woods for comments on an earlier version of this manuscript and R. Scott for use of the centrifuge.

A negatively powered lens in the chameleon

Matthias Ott & Frank Schaeffel*

University Eye Hospital, Department of Experimental Ophthalmology, Roentgenweg 11, 72076 Tübingen, Germany

* To whom correspondence should be addressed

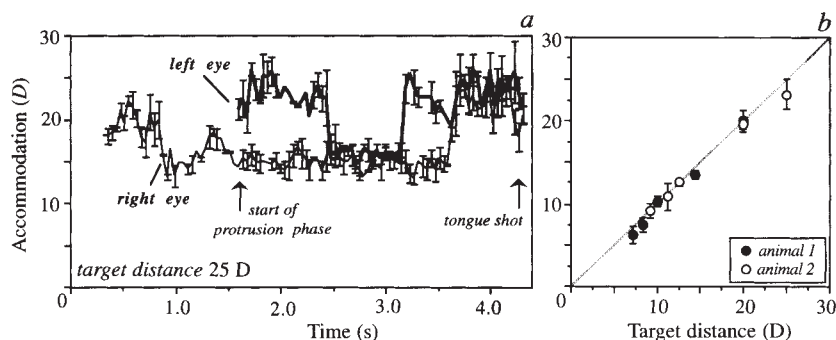
CHAMELEONS are arboreal lizards that spot their prey visually and catch it by highly precise shots with their long sticky tongue. They scan their environment by large-amplitude independent saccadic eye movements; once an insect is detected, the head axis is aligned towards the target ('head tracking'¹, both eyes come forward to fixate the insect and, in a phase called 'initial protrusion'², the sticky tongue is loaded with tension by a special hyoid apparatus³ and subsequently shot out of the mouth with great precision. Lenses placed in front of the eyes produce predictable errors in distance estimation⁴, suggesting that chameleons rely on accommodation cues when measuring the distance to their prey, but focusing has never been measured directly. Using a new technique to measure accommodation⁵, we now show that accommodation is precise enough to serve as the major distance cue. Because accurate focusing requires large retinal images, we have tested image magnification and find that it is higher than in any other vertebrate eye scaled to the same size. This is a result of a unique optical design: unlike other vertebrate eyes, the crystalline lens of the chameleon

has negative refractive power. Although there is a trend among vertebrates to increase corneal power and to decrease lens power with higher visual acuity, only in the chameleon eye has this tendency led to a reversal of the sign of the power of the lens.

We have measured natural accommodation in two tame chameleons (*Chamaeleo dilepis*) sitting on a perch while they caught crickets presented by the investigator. We found that accommodation was very fast (up to 60 dioptres per second) and that accommodative ranges were larger than in other terrestrial vertebrates (up to 45 dioptres); it was also extremely precise. The accommodation response function (Fig. 1b) had a slope of almost exactly one (animal 1, 1.04, $R=0.995$; animal 2, 0.86, $R=0.992$ where R is Pearson's correlation coefficient). Required and measured accommodation did not differ significantly at any distance tested. In contrast to other animals in which accommodation was directly measured and which did not accommodate precisely⁶, chameleons accommodated accurately enough to extract distance information solely from this cue.

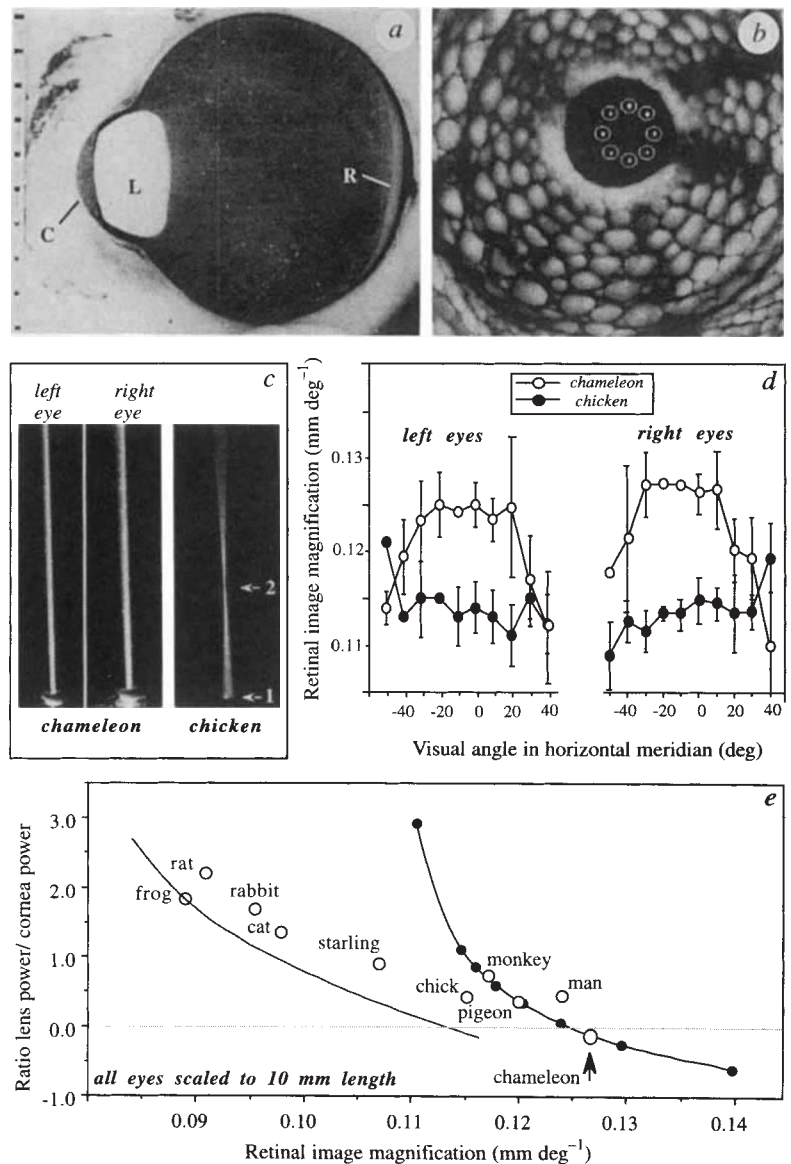
A requirement for precise accommodation is high visual acuity. Visual acuity, in turn, is dependent on retinal image quality and on retinal image size. As the sampling interval of the retinal photoreceptors cannot be made smaller than about 1.6 μm without optical crosstalk between single receptors precluding further resolution⁷, eye size and retinal image size generally increase in vertebrates with increasing visual acuity⁸. However, there are also remarkable differences in eye design to achieve the largest possible retinal image for a given eye size.

FIG. 1 Accommodation in the chameleon eye as functions of time (a) and distance (b). a, The prey cricket was presented at a distance of 4 cm (equivalent to 25 dioptres (D)). Accommodation was independent in both eyes but finally locked together shortly before the tongue shot occurred. At this time, accommodation remained coupled in both eyes even if one eye was covered with an infrared-light-transmitting filter which blocked vision but still permitted measurement (data not shown). Furthermore, accommodation was accompanied by a prominent pupillary near response (linear regressions: animal 1, pupil size (mm) was $2.759 - 0.0286 \times \text{refraction}$ (dioptric value without unit), $R=0.867$, $n=145$; animal 2, pupil size was $3.017 - 0.0389 \times \text{refraction}$, $R=0.879$, $n=158$, $P<0.0001$ in both cases). Measurement noise is illustrated by the standard deviation from three repeated analyses of the record. b, Prey crickets were presented at various distances (plotted in dioptric equivalents) and the amount of accommodation was measured shortly before the tongue shot. Accommodation was very precise for all distances ranging from 4 to 15 cm (25 to 7 dioptres). Error bars represent ± 1 s.d. from 15 to 50 repeated measurements. The resting refraction of the chameleon was difficult to determine because of its continuously active accommodation. From the thickness of the retina (about 235 μm), one would expect a small eye artefact¹² of 3.4 dioptres. The small eye artefact could be neglected because measurements were based on previous calibration with lenses.



METHODS. Using an infrared-sensitive video camera (Canon PI-20PR) with an infrared photoretinoscope (hand-made) attached to a Zeiss F=85 mm, f/1.4 lens, chameleons were video-taped from a distance of 1 m. After previous calibration of the refraction technique with ophthalmic lenses, the slopes of the brightness profiles in the pupils as measured in the individual video frames could be converted into refractions⁵. To ensure precise measurements of the brightness slopes in the pupils, one of us (F.S.) wrote a computer program that recorded 125 video frames digitally on to a virtual disk on a personal computer (25-Hz sampling rate) and automatically fitted the brightness profiles in the pupils by linear regression.

FIG. 2 a, Frozen section of the chameleon eye. The corneal (C) radius of curvature is very small, the surfaces of the lens (L) are quite flat and the thickness of the retina (R) is impressive in the centre but drops off sharply in the periphery. Although the external shape of the lens does not indicate that its power is negative, its internal isoindical shells must be concave. Distance between calibration bars on the left equals 1 mm. b, A computer program written in lattice C located the reflexes created by a circular arrangement of infrared light-emitting diodes (LEDs) on the corneal surface in a highly magnified video image and calculated radius of curvature⁹; the technique was calibrated in a series of four different ball bearings. Measurement variability was less than 0.05 mm (equivalent to 2 dioptres in the chameleon eye); the results did not differ from direct measurements of corneal curvature in frozen sections. Averages for the schematic eye data from the three eyes were (from two animals, homogenous lens model, except for corneal curvature which was measured *in vivo* in eight eyes of four animals) anterior cornea (position/radius of curvature) $0.0/2.225 \pm 0.049$ mm ($n=8$), posterior cornea 0.205 ± 0.035 mm/ 2.02 ± 0.057 mm, anterior lens surface 0.645 ± 0.021 mm/ 4.52 ± 0.17 mm ($n=3$), posterior lens surface 2.600 ± 0.042 mm/ -4.00 ± 0.142 mm ($n=3$), vitreo-retinal surface (position) 9.65 ± 0.0141 mm ($n=3$), photoreceptor layer 9.885 ± 0.163 mm ($n=3$). Refractive indices were taken from the iguana¹³: cornea 1.35, aqueous 1.336, lens 1.4, vitreous 1.336. To achieve emmetropia for the schematic eye model with a homogenous lens index, lens radii of curvature had to be reversed and set at -8.2 mm (anterior surface) and $+8.2$ mm (posterior surface). Pupil size was 2.20 ± 0.11 mm which gives an f number of 3.30, with a posterior nodal distance of 7.264 mm. c, Direct demonstration of negative refractive power of the chameleon lens. The anterior segments of both freshly enucleated eyes were placed on a small ring and immersed in saline rendering corneal refractive power close to zero. The beam of a He/Ne laser entered the dioptric apparatus of the eye (arrow 1) from below. In agreement with our calculations, the beam diverged behind the lens in both eyes of the chameleon, as expected if its power is negative. By contrast, the lens of the chicken focused the beam at a distance of 39 mm. d, Retinal image magnification across the visual field in the chameleon and the chicken. Four infrared LEDs (maximal radiation at 890 nm) arranged in a square of 190 mm side length were placed at a distance of 560 mm from an excised eye and imaged on the retina. The infrared images could be detected through fundal layers by the infrared video camera placed behind the eye; retinal image magnification was determined in highly magnified digitized video frames from simple ray equations¹⁴. The eyes were placed on a rotatable turntable with an angular scale and the image magnification was measured across the horizontal visual field. To validate the technique and to facilitate comparisons between two species, two eyes from a chicken (also with ~ 10 mm axial length) were measured in the same way. In agreement with previous measurements¹⁴, image magnification did not vary much across the visual field in the chicken; a considerable increase in image magnification in the central visual field was only found in the chameleon. e, Comparison of retinal image sizes in various vertebrate eyes all scaled to 10 mm axial length. Vertebrates with high visual acuity have higher retinal image magnification (schematic eye data from refs 8, 15). With increasing relative image size, there is a



decline in the ratio of lens power to corneal power. The ratio becomes minimal in the chameleon, the only animal in which it is negative (-0.1043). However, the ratio is not the only parameter that determines image size. Using the surface position data of the schematic eyes of the various species and a schematic eye construction program¹⁶, we have simulated various combinations of lens and corneal power while keeping the eyes emmetropic. As can be expected from the increase in posterior nodal distance that takes place if the relative power of the cornea is increased⁸, image magnification increased in all cases. Examples of the simulations are shown for the chameleon (right line) and for the frog (left line). Only with the surface position data of the schematic eye of the chameleon could the large image magnification be produced that was actually measured (d).

We have studied the optics of the chameleon eye with schematic eye data derived from frozen sections (Fig. 2a) and *in vivo* measurement of corneal refractive power (Fig. 2b). The result is that the eye would be 32.1 dioptres myopic and the image 1.72 mm in front of the retina; even if the crystalline lens had zero power, the eye would still be 12.2 dioptres myopic with an out-of-focus distance of 0.8 mm. To reconcile the calculated refraction with the measured refraction, there is optically no explanation other than that the power of the crystalline lens is negative. It is clear that the conclusion rests on the validity of the measurements of corneal curvature and axial length. We measured corneal curvature in both eyes of four alert animals (including the two specimens killed for this study) using auto-

mated infrared photokeratometry⁹ (Fig. 2b). The average corneal radius of curvature was only 2.23 ± 0.04 mm, which is 1.4 mm (37%) less than in a 25-day-old chicken with a similar axial length¹⁰. To prove the existence of a negatively powered lens directly, we used an approach similar to one originally designed in 1801¹¹. The anterior segment of the eye (cornea and lens), resting on a small plastic ring with the cornea pointing down, was immersed in a glass container filled with saline. In saline, the refractive power of the cornea is virtually zero owing to the similarity of the refractive indices of the corneal tissue and saline. The remaining refractive power originates from the lens; to measure it, the beam of a helium-neon laser was directed to enter the downward-pointing cornea from below (Fig. 2c,

arrow 1). A few drops of milk were added to the saline so that the path of the beam became visible. The results confirmed our calculations: the chicken lens focused the laser beam at a distance of 39 mm (Fig. 2c, arrow 2), but there was no focal point behind the chameleon lens and there was even some divergence of the beam. To our knowledge, a lens with negative power has not been described before in vertebrates.

Two possible evolutionary pressures may have produced the unusual optical design: (1) a lens with negative power at relaxed accommodation may further extend the range of accommodation and/or (2) a negative lens in combination with a high powered cornea may maximize the relative retinal image size. Although we cannot prove the validity of the first assumption, we have tested the second. Comparing transcleral images in excised chameleon and chicken eyes (both had an axial length of about 10 mm), we found that the retinal image is 15% larger in the centre of the visual field of the chameleon eye than in the chicken, but drops off to a similar size 40 degrees off-axis (Fig. 2d). To evaluate the significance of this observation, we compared retinal image sizes in a variety of vertebrate eyes after scaling them down to similar size (10 mm axial length; Fig. 2e). It can be seen that, for the given eye size, the chameleon indeed has the largest retinal image. The large image size is only achieved because the ratio of the power of the lens to the power of the cornea is lower than in all other vertebrates and even has

a negative value. This phenomenon is not restricted to a single species of chameleons; we have also tested two *Chamaeleo calyptratus* and found a similar optical design with a negatively powered lens. □

Received 2 October; accepted 12 December 1994.

1. Flanders, M. *Vision Res.* **25**, 935–942 (1985).
2. Altevogt, R. & Altevogt, R. Z. *Vergl. Physiol.* **36**, 66–77 (1954).
3. Wainwright, P. C. & Bennett, A. F. *J. exp. Biol.* **168**, 1–21 (1992).
4. Harkness, L. *Nature* **267**, 346–349 (1977).
5. Schaeffel, F., Hagel, G., Eikermann, J. & Collett, T. J. *Opt. Soc. Am.* **A11**, 487–495 (1994).
6. Wagner, H. & Schaeffel, F. *J. comp. Physiol.* **A169**, 515–521 (1992).
7. Miller, W. H. in *Handbook of Sensory Physiology* Vol. VII/6A (ed. Autrum, H.) 69–143 (Springer, Berlin, 1979).
8. Martin, G. in *Perception and Motor Control in Birds* (eds Davies M. N. O. & Green, P.) 5–30 (Springer, Berlin, 1994).
9. Schaeffel, F. & Howland, H. C. *J. comp. Physiol.* **A160**, 375–384 (1987).
10. Schaeffel, F. & Howland, H. C. *Clin. vis. Sci.* **3**, 83–99 (1988).
11. Young, T. *Phil. Trans. R. Soc.* **19**, 23–88 (1801).
12. Glickstein, M. & Millodot, M. *Science* **192**, 605–606 (1970).
13. Citron, M. C. & Pinto, L. H. *Vision Res.* **13**, 873–876 (1973).
14. Schaeffel, F., Glasser, A. & Howland, H. C. *Vision Res.* **28**, 639–657 (1988).
15. Hughes, A. in *Handbook of Sensory Physiology* Vol. VII/5 (ed. Crescitelli, F.) 613–756 (Springer, Berlin, 1977).
16. O'Keefe, L. P. & Coile, D. C. *Ophthalm. Physiol. Opt.* **8**, 97–100 (1988).

ACKNOWLEDGEMENTS. This study was supported by the German Research Council and by the Schilling-Stiftung from the German Stifterverband. We are grateful to the government department for National Parks and Wildlife of Zimbabwe for the CITES permit for *Chamaeleo dilepis*, K. Leuschner for his help at the collection sites, and H. Howland, T. Collett and M. Land for comments on the manuscript.

Colour constancy influenced by contrast adaptation

Michael A. Webster & J. D. Mollon*

Department of Psychology, University of Nevada, Reno, Nevada 89557, USA

* Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB, UK

VISUAL sensitivity is controlled by at least two distinct types of adaptation: light adaptation adjusts sensitivity to the mean luminance and colour in the stimulus¹, and contrast adaptation adjusts sensitivity to the variations in luminance and colour^{2–5}. Light adaptation is thought to be important in maintaining the perceived colour of objects despite changes in illumination ('colour constancy'), compensating for the mean changes in the light reflected from scenes under different illuminants⁶. But for naturalistic colour signals, we show here that changes in an illuminant can also alter colour contrasts in images (how colours are distributed around the mean) enough to alter the state of contrast adaptation. Thus perceived colour under different illuminants may also be noticeably influenced by contrast adaptation.

We used a matching task to examine how light adaptation and contrast adaptation combine to influence colour appearance (Fig. 1). Observers fixated between two fields; in one field they adapted either to a static chromaticity or to 1 Hz modulations around a particular chromaticity. (Using an electrical analogy, we refer to the former as a DC stimulus and the latter as DC+AC.) The perceived colour of test chromaticities in the adapting field was then matched by adjusting chromaticity in the second, neutral-adaptation field. Figure 2 shows the effects of adapting to four different DC chromaticities or to modulations around each DC value along two different chromatic directions (at nominal angles of 45–225° or 135–315° within an L-M cone versus S cone colour plane⁷). Before adaptation the four DC chromaticities appear a moderately saturated blue (Fig. 2a), purple (Fig. 2b), yellow-green (Fig. 2c) or orange (Fig. 2d). However, adaptation to each static chromaticity causes it to appear nearly (but not completely) achromatic, and produces concomitant shifts in the appearance of test stimuli. Thus test stimuli that all appeared blue (Fig. 2a) before adaptation, are matched after

adaptation by a wide range of hues bracketing white. These shifts occur independently along the S and L-M axes, consistent with independent sensitivity changes within each cone class that rescale the cone signals so that the mean response is (nearly) the same before and after adaptation (von Kries adaptation⁸). Adaptation to the DC+AC stimuli produces the same mean colour shifts, but in addition reduces the perceived contrast of test stimuli relative to the DC match. The saturation losses are always largest along the colour direction defining the adapting contrast. (For example, after adaptation to each DC chromaticity, the 135–315° modulations all appear to vary between blue and yellow, and produce the largest saturation losses on that axis.) Saturation losses selective for these directions imply an interaction between different cone classes at sites central to the sites of light adaptation^{5,9}. Our results suggest that the DC and AC adapting components produce independent and qualitatively different changes in colour appearance. Light adaptation rescales the cone signals available to subsequent levels, but otherwise appears to have little influence on the properties of contrast adaptation, and there is no evidence of contrast adaptation to the static adapting stimulus. Conversely, the addition of large AC components does not significantly alter the mean perceived colour relative to the DC match, suggesting that the response to chromatic contrast up to the sites of light adaptation is roughly linear (or symmetrical for opposite excursions from the mean).

We examined the influence of illuminant shifts on contrast adaptation, first by modelling the visual response to an illuminant change and then by testing the model empirically. The model was based on two stages of adaptation: an initial von Kries scaling of the cone signals to the mean luminance and chromaticity in the image, followed by losses in contrast sensitivity that are selective for the directions in colour space along which colour signals in the image vary. The observed selectivity of contrast adaptation for any arbitrary colour direction can be modelled either as response changes in multiple post-receptoral channels that each receive a different combination of cone inputs^{5,9}, or as adaptation-dependent interactions between a small set of channels that alter their colour selectivity^{5,10,11}. The latter model, which we assume below for convenience, derives from the hypothesis that contrast adaptation decorrelates the responses across visual channels in order to encode image contrasts more efficiently².

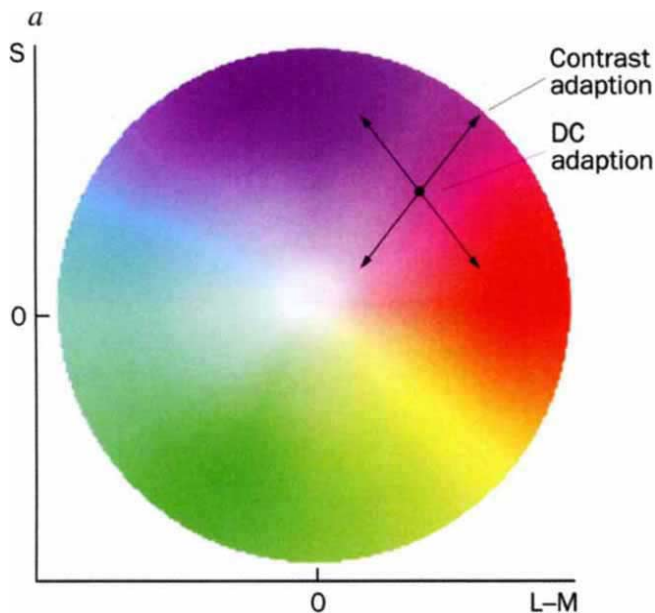
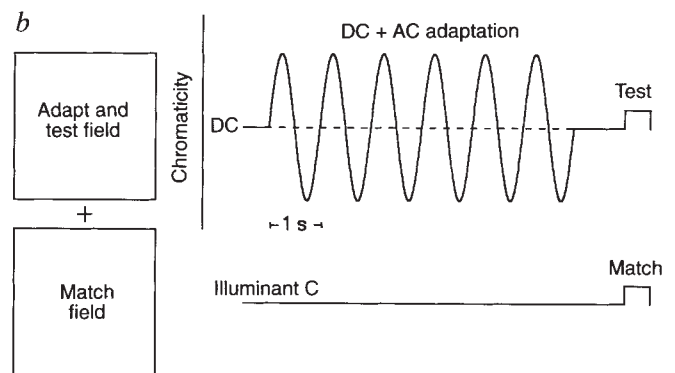


FIG. 1 a, A schematic illustration of the equiluminant colour plane used in this study. Stimuli varying along a vertical axis differ only in the excitation of the short-wave cones, whereas stimuli varying along a horizontal axis differ only in the difference of long-wave and middle-wave cone excitations. We refer to DC adaptation when the subject adapts to a static coloured field (solid point in the figure) and to contrast adaptation when the adapting field varies in chromaticity around the DC colour (two examples are indicated by the double-headed arrows).

It is evident that very large changes in contrast could be realized by an arbitrary choice of illuminants and reflectance spectra, but less obvious whether significant contrast changes can also occur when illuminants and surfaces are constrained to have the gently varying spectra of natural stimuli (though see ref. 12). To examine this, we followed several previous studies (such as refs 13, 14) by restricting illuminants to simulated phases of daylight¹⁵, and surfaces to simulated Munsell chips¹⁶. Figure 3a, b shows a pair of examples in which we model the effects on a set of surfaces of shifting the daylight illuminant from 4,800 K to 10,000 K or *vice versa*. In each case the surfaces were chosen to form a distribution of equiluminant colours under the first illuminant with a mean chromaticity equal to the illuminant chromaticity, but with a strong bias along the S axis in the distribution's range. Figure 3c, d shows plots of the predicted matches following von Kries adaptation to the distribution means. von Kries scaling compensates for most of the difference between the two distributions, but cannot discount the illuminant completely^{17,18}. The shift from 4,800 K to 10,000 K tilts the initial distribution off the S axis (90°) to an axis of 100°. The converse shift tilts the distribution from 90° to 77°. In either case the illuminant change has biased the colour direction of the distributions, and should thus alter the state of contrast adaptation. In Fig. 3e, f we model contrast adaptation as a sensitivity loss selective to each axis, by decorrelating and normalizing the responses to the L-M and S contrasts using the algorithm of ref. 10. Colour signals that matched across the two illuminants at the level of light adaptation no longer match, because they are embedded within different contrast distributions and are thus biased by contrast adaptation in different ways. For example, the chromaticities defined by the pair of ellipses in Fig. 3a should give rise to the same set of matches if the visual system adjusts to the illuminant change only through von Kries adaptation. (Thus both ellipses map on to the same circle in Fig. 3c.) Yet because of contrast adaptation, matches to these chromaticities instead plot as ellipses whose minor axes are oriented along the primary axes of the distributions, reflecting the selective sensitivity loss to the different adapting colour directions.



b, Adaptation effects were assessed with an asymmetrical matching procedure. Observers viewed a colour monitor with both eyes and fixated between two 2° fields (0.4° apart). Narrow black borders delimited each field from a white (equivalent to Illuminant C) surround of the same luminance (27.5 cd m⁻²). Adapting stimuli were presented in the upper field, and consisted of a static colour (the DC chromaticity, chosen to induce a change in light adaptation), or 1 Hz sinusoidal modulations of chromaticity around the DC value (DC + AC, chosen to induce changes in both light adaptation and contrast adaptation). After 3-min initial adaptation, a test chromaticity was presented for 500 ms in the adapting field, and then continued interleaved with 7.5-s re-adaptation intervals (with only the DC adapting component present 1 s before and 0.5 s after each test). Observers matched the perceived colour of the test by adjusting the chromaticity of a matching stimulus presented simultaneously with the test, but in the lower, neutral-adaptation (Illuminant C) field.

We measured the empirical colour changes produced by adaptation to the four distributions illustrated in Fig. 3a, b, again using the matching procedure. Test stimuli included the mean chromaticity plus 16 chromaticities spanning the ellipses illustrated for each distribution. The observer adapted either to the static mean chromaticity of each distribution (DC), or to successive random samples from each distribution drawn every 200 ms (DC + AC). The latter simulates for a single retinal locus the temporal modulations of chromaticity that might arise from very rapid and spatially random eye movements over the image. Models of colour constancy have suggested that eye movements could allow spatially local mechanisms to light adapt to the mean colour signal in the image, if light adaptation is slow enough to integrate over many fixations (for example, ref. 19). However, such eye movements should also provide a potent stimulus for contrast adaptation, for we have observed strong contrast adaptation over a wide range of modulation rates, from higher than fixation changes could generate²⁰, to many times lower than are required to maintain light adaptation to the mean chromaticity of our stimuli. (Although we consider here only temporal contrasts, contrast adaptation to spatially distributed patterns (such as gratings) is well established², and appears to induce similar changes in colour appearance (for example, refs 21, 22)).

Figure 4 shows the colour changes produced by adaptation to each distribution. Colour matches following adaptation to the distribution mean are again closely approximated by incomplete von Kries scaling, that is, the matches fall roughly on a circle centred slightly off the origin. Adaptation to the actual samples from the distributions produces a pronounced contrast adaptation effect (in addition to the DC colour change), distorting into ellipses the matches to the nominal circle of test chromaticities. The orientation of each ellipse is close to the orientation predicted in Fig. 3e, f. Thus the illuminant change induces significantly different contrast adaptation effects that are in qualitative agreement with the model.

Our results suggest that the state of contrast adaptation will often change when the illumination changes, and this may both limit and promote the constancy of colour appearance. On the

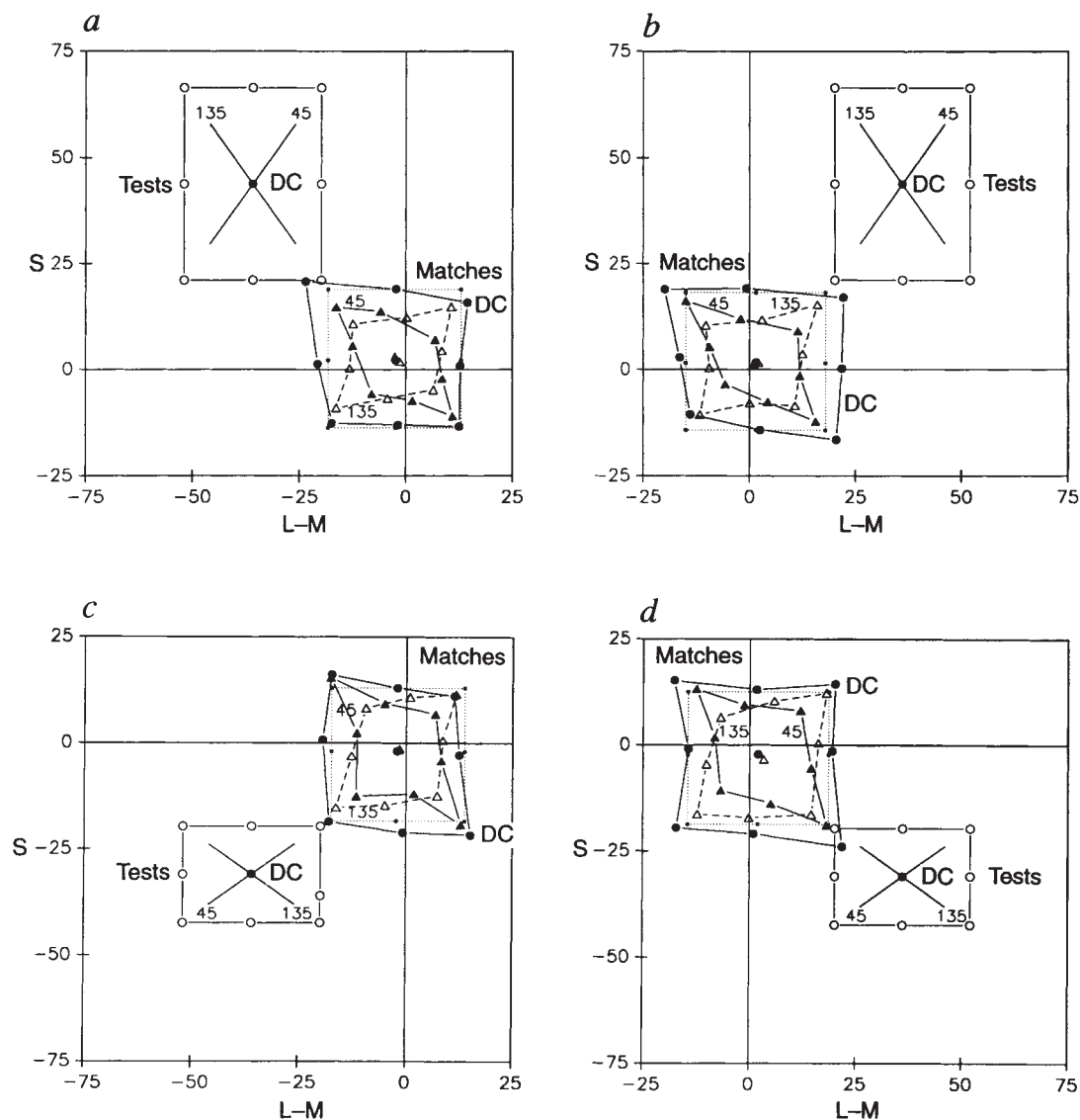
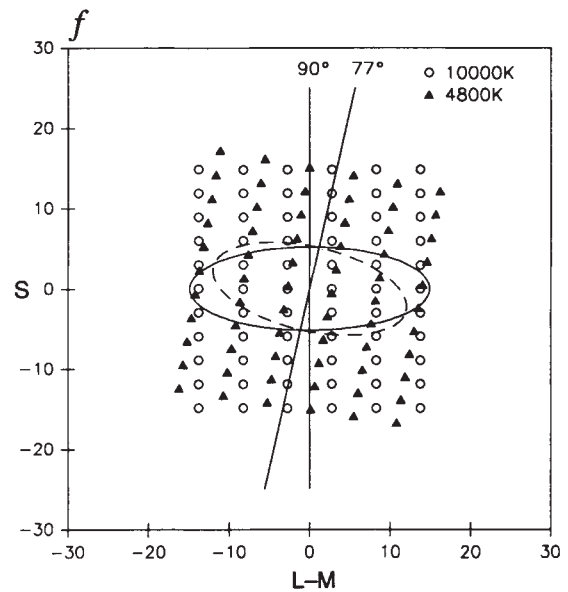
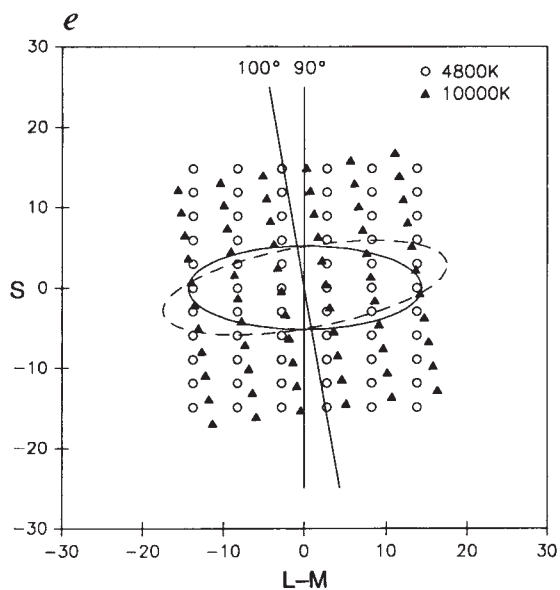
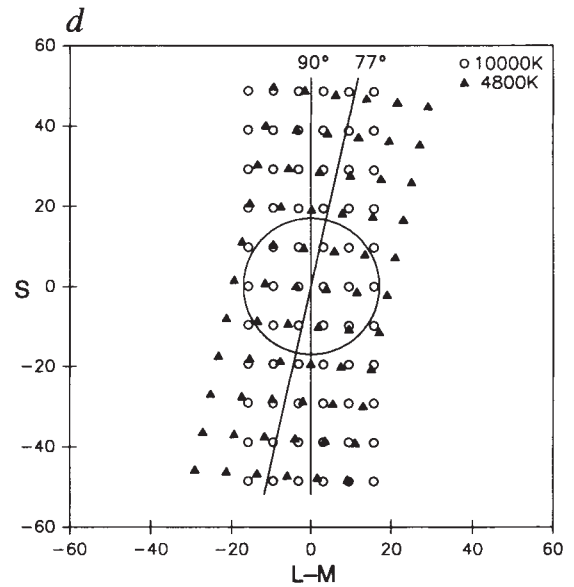
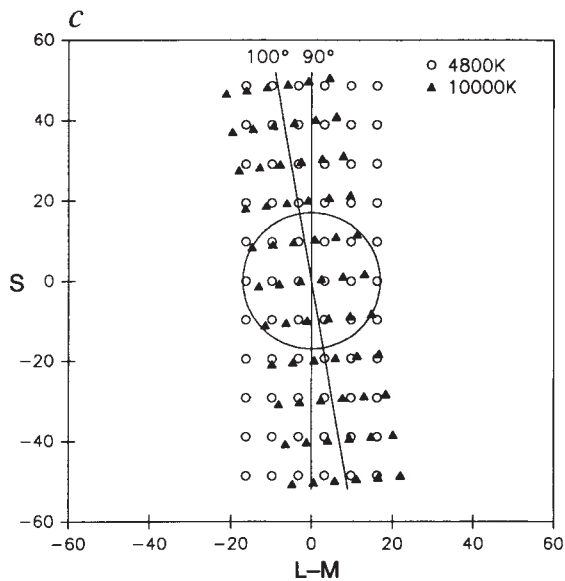
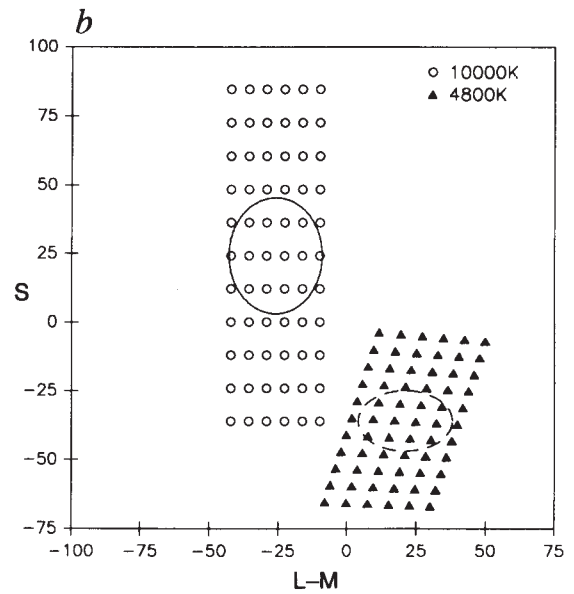
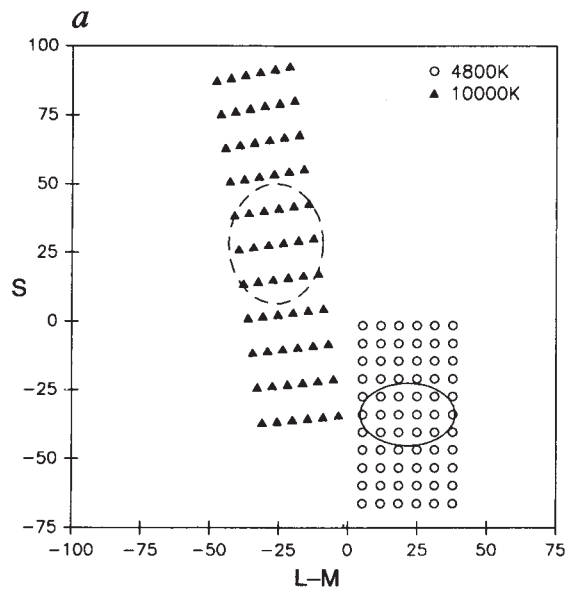


FIG. 2 Matches to test stimuli (open circles) following adaptation to a static colour (DC, filled circles), or to modulations around the DC colour (DC + AC, triangles). All stimuli were equiluminant (27.5 cd m^{-2}) and are plotted within a colour plane⁷ defined by an L-M axis, representing the relative excitation of L versus M cones to each chromaticity, and an S axis, representing S cone excitation. The two axes intersect at an origin corresponding to the chromaticity of Illuminant C, and were scaled for equal contrast sensitivity at the origin (based on measured thresholds). Unit distances along the S and L-M axes correspond to cone contrasts of 0.010 (S), 0.00151 (M) or 0.00079 (L). Each panel plots the matches for DC or DC + AC adapting colours centred in a different quadrant of the S versus L-M plane. AC modulations around each DC varied over a range of $\pm 48 \times$ threshold and varied along nominal angles of $45\text{--}225^\circ$

(yielding matches shown by filled triangles) or $135\text{--}315^\circ$ (matches shown by open triangles). (Test and adapting stimuli in different quadrants were scaled *a priori* to compensate for differences in von Kries adaptation.) Dotted lines/small symbols plot the matches predicted by von Kries adaptation, (based on rescaling each cone's signals according to the match to the DC adapting colour). The cone-specific scaling predicts that changes in perceived colour along the S axis do not depend on the level of L versus M cone excitation (or *vice versa*). This is consistent with the observed DC adaptation effects, but not with the matches following DC + AC adaptation, which reveal pronounced interactions between colour changes along the two axes. Results reported are the average of 3 or more matches to each test for author M.W. All measurements were confirmed on a second, naive observer.

FIG. 3 Simulations of adaptation to an illuminant change. Illuminants were approximated using the first three basis functions derived from daylight spectra by ref. 15. Surface reflectance spectra were simulated with the first three basis functions derived by ref. 16 for Munsell chips. a, Chromaticities of lights reflected from a set of chips viewed under 4,800 K daylight (circles), or the same set under 10,000 K daylight (triangles). The surfaces were chosen so that under the 4,800 K illuminant, the reflected colour signals form an equiluminant distribution centred on the illuminant chromaticity and biased along the S axis (so that the ratio of S to L-M contrasts = 3, following von Kries scaling). (The small effect that the illuminant shift has on luminance is not shown). b, The converse example in which the S-biased distribution is defined for the 10,000 K illuminant (circles) and then viewed under the 4,800 K illuminant (triangles). c, d, Matches to each chip predicted by von Kries

adaptation. Each cone's responses are rescaled by the ratio of the mean excitation (to each distribution) to the reference excitation (to Illuminant C, whose chromaticity plots at the origin). Solid lines show the dominant axis of each distribution. e, f, Matches predicted by contrast adaptation to the von Kries-scaled cone signals. The adaptation was simulated with the algorithm of ref. 10, which rescales sensitivity along the dominant and orthogonal axes of the distributions until (we assume here) responses along the S and L-M axes are decorrelated and normalized to an arbitrarily chosen variance ($\sigma = 9.5$). The matches predicted are consistent with the colour changes that contrast adaptation induces in moderate test contrasts^{4,10}, but probably overestimate changes at the extremes of the distributions, for contrast adaptation has much less effect on high contrast stimuli than predicted by the model's divisive gain control^{5,24}.



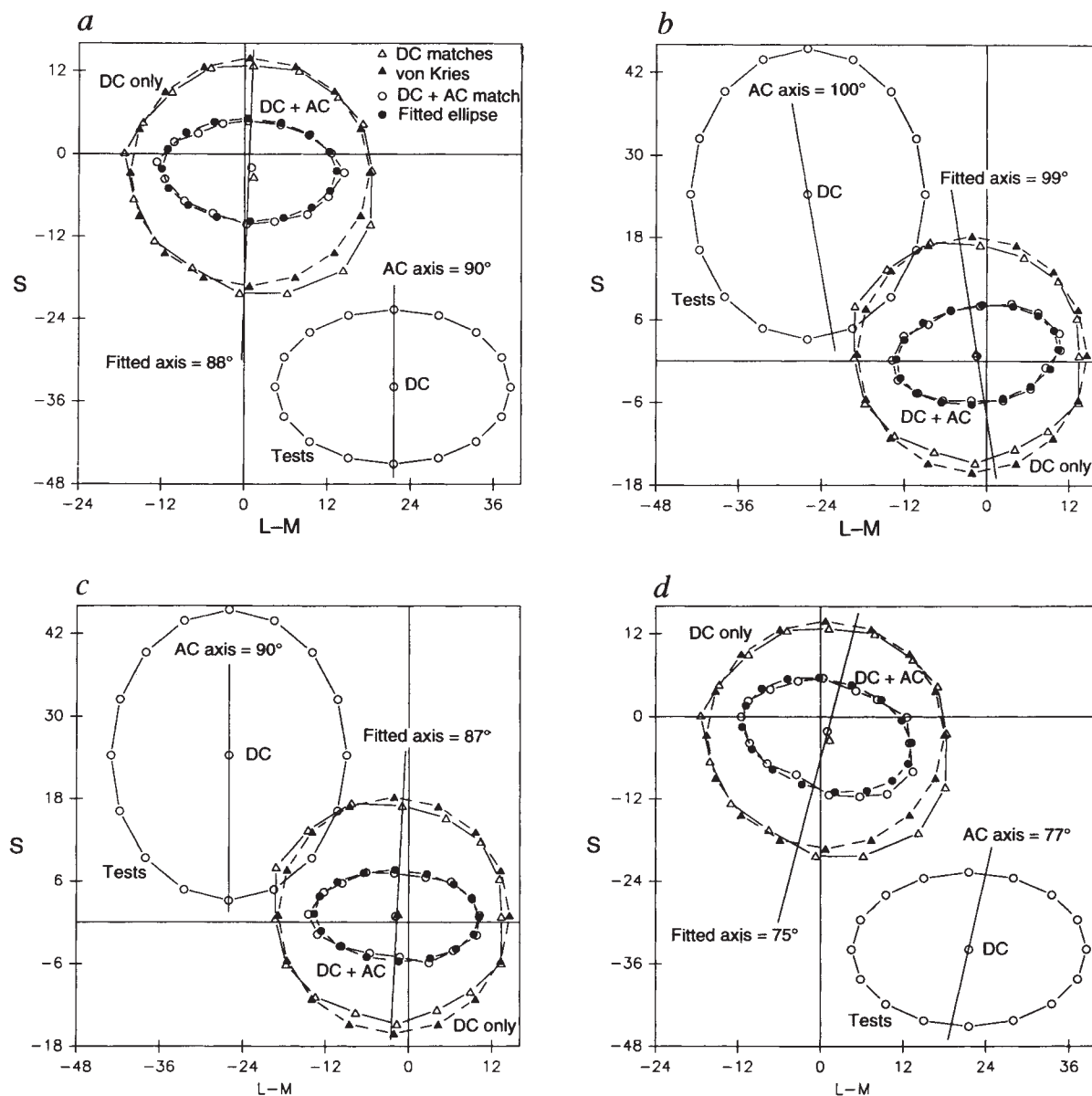


FIG. 4 Empirical matches following adaptation to the four colour distributions shown in Fig. 3a, b. Matches were made after adapting to the distribution mean (DC, open triangles), or successive random samples from each distribution (DC + AC, open circles). Filled triangles plot the matches predicted by von Kries adaptation. Filled circles are predicted matches derived by divisively scaling the von Kries coordinates along

two orthogonal axes to find the least-squares fit to the observed matches. The angles of the two axes were covaried to estimate the best-fitting adapting axis (solid lines). a, b, Matches for the initial S distribution under 4,800 K or the same surfaces under 10,000 K. c, d, Matches for the initial S distribution under 10,000 K or the same surfaces under 4,800 K.

one hand, contrast adaptation biases colour appearance relative to the axes of the adapting distributions, and thus it cannot undo tilts that different illuminants produce in these axes. On the other hand, contrast adaptation rescales apparent contrast according to the range of signals in the stimulus array, and this partially compensates for changes in the colour range introduced by changes in the illuminant or in viewing conditions (for example fog or rain²³). □

Received 17 October; accepted 27 December 1994.

1. Stiles, W. S. *Proc. natn. Acad. Sci. U.S.A.* **45**, 100–114 (1959).
2. Barlow, H. B. in *Vision: Coding and Efficiency* (ed. Blakemore, C.) 363–375 (Cambridge Univ. Press, Cambridge, 1989).
3. Krauskopf, J., Williams, D. R. & Heeley, D. W. *Vision Res.* **22**, 1123–1131 (1982).
4. Webster, M. A. & Mollon, J. D. *Nature* **349**, 235–238 (1991).
5. Webster, M. A. & Mollon, J. D. *Vision Res.* **34**, 1993–2020 (1994).
6. Pokorny, J., Shevell, S. K. & Smith, V. C. in *Vision and Visual Dysfunction Vol. 6: The Perception of Colour* (ed. Gouras, P.) 43–61 (Macmillan, London, 1991).

7. MacLeod, D. I. A. & Boynton, R. M. *J. opt. Soc. Am.* **69**, 1183–1186 (1979).
8. von Kries, J. *Festschrift der Albrecht-Ludwigs-Universität (Fribourg, 1902)*, translated in *Sources of Color Science* (ed. MacAdam, D. L.) (MIT Press, Cambridge, 1970).
9. Krauskopf, J., Williams, D. R., Mandler, M. B. & Brown, A. M. *Vision Res.* **26**, 23–32 (1986).
10. Atick, J. J., Li, Z. & Redlich, A. N. *Vision Res.* **33**, 123–129 (1993).
11. Zaidi, Q. & Shapiro, A. G. *Biol. Cybern.* **69**, 415–428 (1993).
12. Worthey, J. A. *J. opt. Soc. Am.* **72**, 74–82 (1982).
13. Brainard, D. H. & Wandell, B. A. *J. opt. Soc. Am.* **9**, 1433–1448 (1992).
14. Arend, L. E. *J. opt. Soc. Am.* **A10**, 2134–2147 (1993).
15. Judd, D. B., MacAdam, D. L. & Wyszecki, G. *J. opt. Soc. Am.* **54**, 1031–1040 (1964).
16. Cohen, J. *Psychon. Sci.* **1**, 369–370 (1964).
17. MacLeod, D. I. A. in *Central and Peripheral Mechanisms of Colour Vision* (ed. Ottoson, D. & Zeki, S.) (Macmillan, London, 1985).
18. Worthey, J. A. & Brill, M. H. *J. opt. Soc. Am.* **A3**, 1708–1712 (1986).
19. D'Zmura, M. & Lennie, P. *J. opt. Soc. Am.* **A3**, 1662–1672 (1986).
20. Yarbus, A. L. *Eye Movements and Vision* (translated Haigh, B.; ed. Riggs, L. A.) (Plenum, New York, 1967).
21. Flanagan, P., Cavanagh, P. & Crassini, B. *Invest. Ophthalmol. Suppl.* **30**, 130 (1989).
22. Webster, M. A. & Mollon, J. D. *J. opt. Soc. Am.* **A10**, 1332–1340 (1993).
23. Brown, R. O. & MacLeod, D. I. A. *Invest. Ophthalmol. Suppl.* **32**, 1214 (1991).
24. Georgeson, M. A. *Spatial Vision* **1**, 103–112 (1985).

ACKNOWLEDGEMENTS. This work was supported by the SERC.

Scatter factor/hepatocyte growth factor is essential for liver development

Claudia Schmidt, Friedhelm Blatt,
Stefanie Goedecke, Volker Brinkmann*,
Wolfgang Zschiesche*, Melanie Sharpe†,
Ermanno Gherardi† & Carmen Birchmeller‡

Max-Delbrück Laboratorium in der Max-Planck-Gesellschaft,
Carl-von Linné-Weg 10, 50829 Köln, Germany

* Max-Delbrück Centrum für Molekulare Medizin,
Robert-Roessle-Strasse 10, 13122 Berlin, Germany

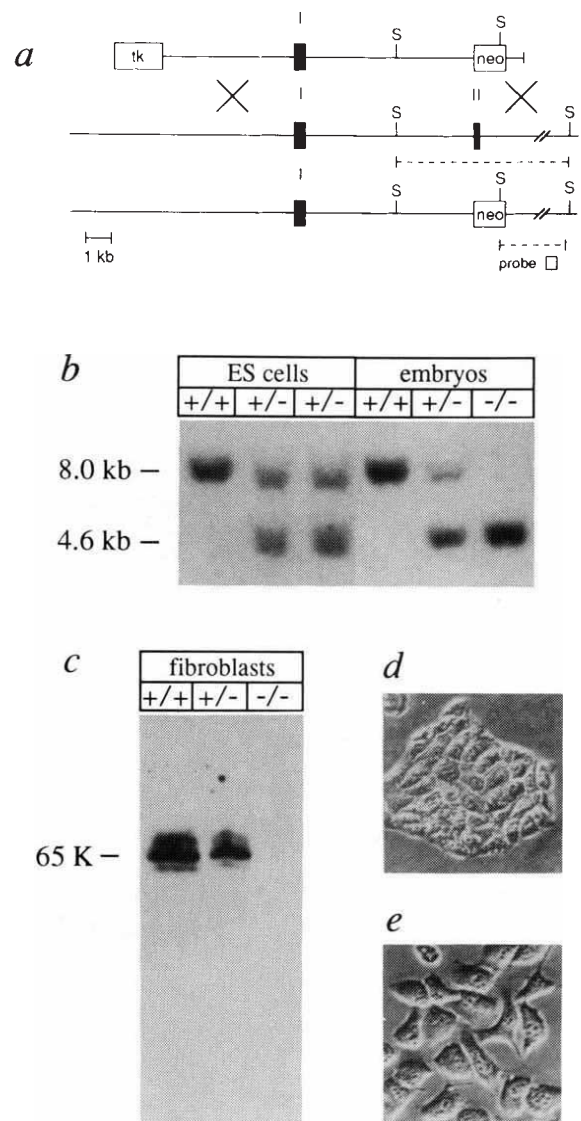
† ICRF Cell Interactions Laboratory,
Cambridge University Medical School, MRC Centre, Hills Road,
Cambridge CB2 2QH, UK

‡ To whom correspondence should be addressed

POLYPEPTIDE growth factors are important effectors of cell growth and differentiation *in vitro* and are thought to be critical for processes such as specification of cell fate, tissue growth and organogenesis *in vivo*. Scatter factor¹⁻³/hepatocyte growth factor^{4,5} (SF/HGF) is the prototype of an emerging family of growth factors that resemble in their domain structure and mechanism of activation the blood proteinase plasminogen^{6,7}. The cellular responses of SF/HGF are mediated by the c-Met tyrosine kinase receptor⁸⁻¹⁰. Here we report that mice lacking SF/HGF fail to complete development and die *in utero*. The mutation affects the embryonic liver, which is reduced in size and shows extensive loss of parenchymal cells. In addition, development of the placenta, particularly of trophoblast cells, is impaired. Thus, SF/HGF is essential for the development of several epithelial organs.

SF/HGF has multiple activities on target cells in culture^{1-5,11-14} and has been implicated in mesenchymal-epithelial interactions during organ development^{15,16}. To understand the physiological role of the factor, we produced embryonic stem (ES) cells in which exon 2 of the SF/HGF gene, which encodes amino acids 31 to 84 and thus part of a protein domain essential for receptor binding¹⁷, was replaced with a neomycin-resistance (*neo*) gene (Fig. 1a). Healthy and fertile mice carrying the mutation in a heterozygous state were generated from two independent ES cell clones. Matings between heterozygous animals produced no live SF/HGF^{-/-} offspring, implying that mutant embryos die during development (Table 1). To determine the time of death, embryos from different stages were collected and genotyped. Up to day 13 of embryogenesis (E13), the expected frequency of 25% homozygous mutant embryos was observed but, at stages E14.5 and E16.5, the proportion of viable SF/HGF^{-/-} embryos declined to 12 and 2%, respectively. Often the SF/HGF^{-/-} embryos were found dead *in utero* (as judged by the absence of heartbeat or by signs of resorption).

Immunoblot analysis of fibroblast cultures established at E12.5 from wild-type, heterozygous and homozygous embryos showed that homozygous cells lack SF/HGF protein (Fig. 1c) and MDCK-scattering activity (Fig. 1d,e), the most sensitive functional assay for SF/HGF. SF/HGF protein and activity were readily detected in medium conditioned by fibroblasts from heterozygous and wild-type embryos (Fig. 1c,e). Thus we have



from genomic DNA of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) embryos. **c**, Immunoblot of medium conditioned by fibroblasts from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) E12.5 embryos with anti-SF/HGF rabbit IgG. **d** and **e**, Appearance of MDCK colonies in the presence of medium conditioned by fibroblasts from homozygous (**d**) and wild-type (**e**) embryos.

METHODS. Genomic SF/HGF DNA isolated from a library of the ES cell line E14 TG2A in λ 2001 was used to construct the targeting vector. In this, exon 2 of the SF/HGF gene was replaced by the *neo* gene and the herpes simplex thymidine kinase gene was fused at the 5' end²⁵. The targeting vector was inserted in E14.1 cells²⁶ by electroporation and homologous recombination events were enriched for by selection with G418 and gancyclovir and identified by PCR. The structure of the mutant locus and the presence of a single insertion event in ES cell colonies were verified by Southern hybridization. Two independently mutated ES clones were used to produce mutant mice by blastocyst injection²⁷. Phenotypes were analysed in both C57BL/6/129 hybrid background and in inbred 129 background. For immunoblot analysis, fibroblast cultures were established from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) from E12.5 embryos and propagated in 10% FCS in DMEM. Cells at passage-5 were grown to confluence, washed twice with PBS and incubated in serum-free DMEM for 3 days. 100 μ l heparin-Sepharose CL beads (50%, v/v) were added to the medium from $\sim 2.5 \times 10^7$ cells to partially purify and concentrate the factor. Samples were separated in a 12% polyacrylamide gel under non-reducing conditions, transferred on PVDF membrane and SF/HGF was detected using rabbit IgG anti-mouse SF/HGF (F1B3), HRP-conjugated goat anti-rabbit IgG and ECL substrate (Amersham). Fibroblast supernatants were assayed for MDCK scattering activity as described³.

FIG. 1 Outline of the strategy used to disrupt the SF/HGF gene and effect of the mutation. **a**, Schematic representation of the targeting vector (top line), the wild-type SF/HGF allele (middle line) and the mutated allele (bottom line). The open box corresponds to the sequence used as a probe for Southern hybridization shown in **b**. The dashed lines indicate the size of the *Stu*I fragments detected in wild-type and mutant genomic DNA. **b**, Southern blot analysis of genomic DNA from wild-type (+/+) and mutated (+/-) ES cells (clones 24 and 35) and

introduced a null mutation into the SF/HGF gene which results in embryonic lethality between E13 and E16.5.

Viable SF/HGF^{-/-} embryos at E14.5 were generally normal in external appearance; they were pink in colour and well vascularized (Fig. 2*a, b*). When compared to +/+ or +/- littermates, a slight reduction in body weight (averaging 17%) and thus in

overall size was apparent (Fig. 2*a, b*). This was not observed in E12.5 embryos. In addition, a small proportion of the mutant embryos showed irregular heartbeat and internal bleeding. These changes might be associated with embryonal death and onset of resorption. When the internal organs were inspected, we found that the livers of SF/HGF embryos were severely reduced in

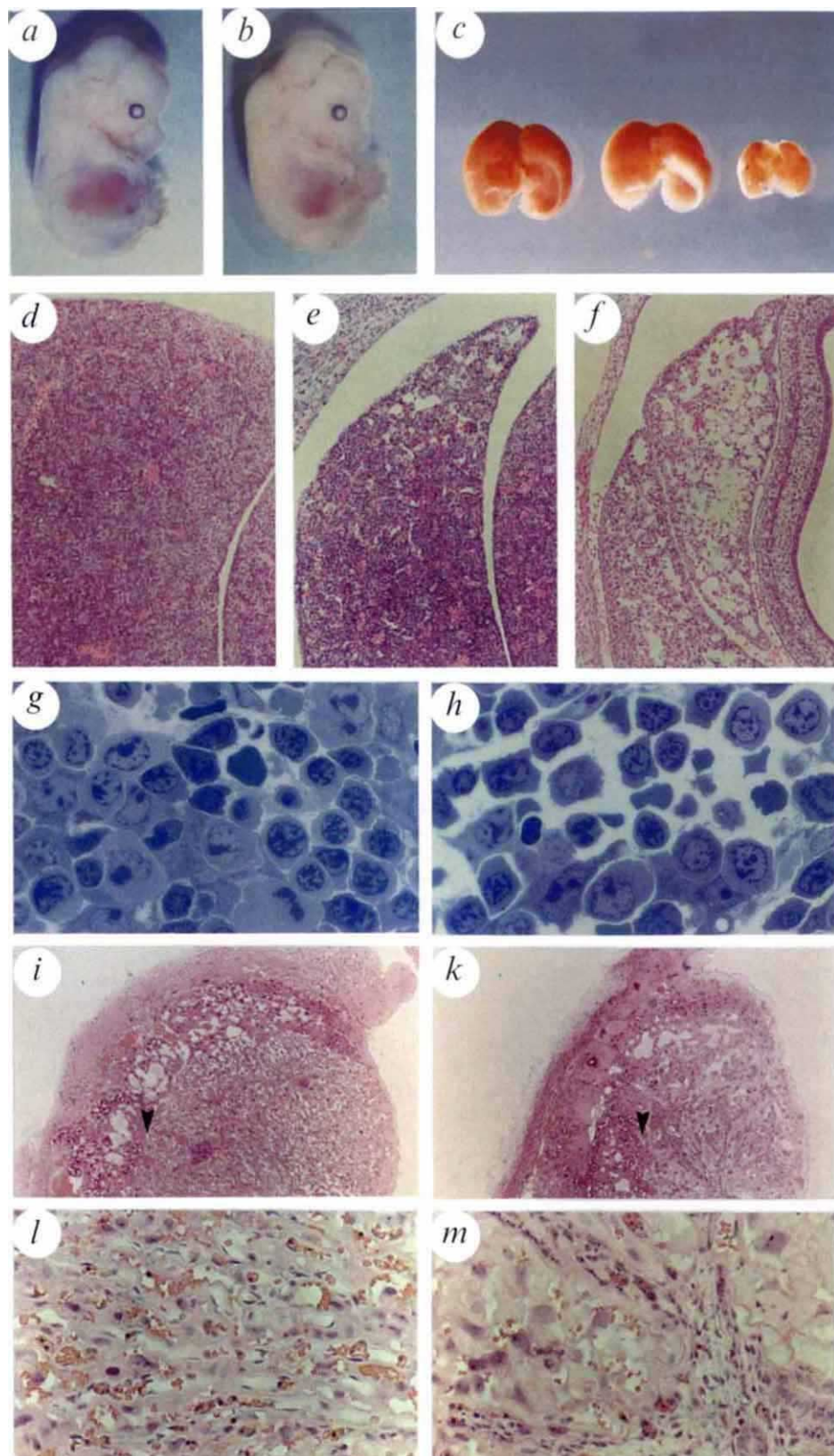


FIG. 2 Morphological and histological appearance of embryos mutant for the SF/HGF gene. *a*, External appearance of SF/HGF^{+/-} and *b*, SF/HGF^{-/-} embryos at E14.5 ($\times 3.7$). *c*, External appearance of livers from wild-type (left), SF/HGF^{+/-} (middle) and SF/HGF^{-/-} (right) embryos at E14.5 ($\times 9$). *d-f*, Haematoxylin-and-eosin-stained sections of wild-type (*d*) and SF/HGF^{-/-} (*e* and *f*) embryos at E14.5 demonstrating enlarged sinusoids and tissue damage in the ventral area (*e*) and severe loss of cellularity in the liver parenchyma ($\times 30$). *g, h*, Semithin sections of livers from E14.5 wild-type (*g*) and SF/HGF^{-/-} (*h*) embryos showing loosened structure of the liver parenchyma ($\times 1,100$). *i-m*, Haematoxylin-and-eosin-stained sections of wild-type (*i, l*) and SF/HGF^{-/-} (*k, m*) placentas showing defects in development of the labyrinth layer (*i, k*; $\times 31$; *l, m*; $\times 230$). Arrows (*i, k*) point towards the junctional zone between labyrinth and spongiotrophoblast layer.

METHODS. For haematoxylin and eosin staining, wild-type and SF/HGF^{-/-} embryos (E14.5) were embedded in Technovit 7100 (Hereaus Kulzer GmbH) and 8- μ m sections were used for staining. For semithin sections, dissected livers were fixed in 2.5% glutaraldehyde and post-fixed in 1% OsO₄; 0.5- μ m sections were stained with fuchsin and Azure II.

size compared to livers of wild-type littermates (Fig. 2c). The reduction was first noted at E12.5 (40%) and was more pronounced at E14.5 (55%). The ratio of liver to body weight at E14.5 differed by a factor of 2 in SF/HGF^{-/-} and wild-type embryos and provided a useful predictor of the SF/HGF^{-/-} genotype. A small but reproducible reduction (9%) of the liver size was noticeable in SF/HGF^{+/-} embryos at E14.5. Histological analysis of SF/HGF^{-/-} embryos at E14.5 showed variable degrees of liver damage. This was not observed in wild-type and heterozygous embryos of the same stage (Fig. 2d) or in younger (E12.5) homozygous embryos. The most consistent abnormality was a loosened liver structure with enlarged sinusoidal spaces (Fig. 2e) and dissociation of the parenchymal cells (Fig. 2h). The dissociated cells often showed signs of apoptosis, including typical nuclear fragmentation which was apparent after staining the nuclei with the intercalating DNA dye Hoechst 33258 (Fig. 3c, d). A severe loss of cellularity was observed in a number of embryos (Fig. 2f) and, typically, the ventral side of the liver was most affected. Cytokeratin immunohistochemistry showed that the hepatocytes in the liver of SF/HGF^{-/-} mice were abnormal in morphology and reduced in number (Fig. 3a, b). In contrast, the morphology of the cells lining the sinusoids, identified by vimentin immunohistochemistry, was not altered even in livers with a severe loss of cellularity (results not shown).

A medium containing growth factors which supports selectively the growth of epithelia¹⁸ was used to culture primary hepatocytes from E14.5 embryos. Although the number of hepatocytes from livers of SF/HGF^{-/-} embryos was severely reduced compared to the number obtained from wild-type livers, no change in growth or survival of the residual hepatocytes was apparent. Probably, other growth factors compensate for the absence of SF/HGF under these *in vitro* conditions.

Because in embryos the liver is the major haematopoietic organ, we analysed erythropoiesis by benzidine histochemistry. In homozygous mutant livers with enlarged sinusoidal spaces numerous erythropoietic islands were present; occasionally their density was even higher than in the wild-type livers (Fig. 3f). Haematopoiesis, however, was affected in livers that had a severe loss of cellularity. In half of the E14.5 SF/HGF^{-/-} embryos and in a single live E16.5 SF/HGF^{-/-} embryo, erythrocyte counts on embryonal blood were reduced. The relative proportion of enucleated erythrocytes and of myeloid cells, however, were similar in wild-type, heterozygous and homozygous embryos. Thus, the effect of the SF/HGF mutation on haematopoiesis probably reflects the reduced size of the liver and the extensive cell death and is considerably less severe than the defects associated with the *c-myb* or *Rb-1* phenotypes¹⁹⁻²¹. Similarities between the SF/HGF^{-/-} and *c-jun*^{-/-} animals²² are apparent: *c-jun*^{-/-} embryos die within the same time range in development; liver cells from *c-jun*^{-/-} embryos are dissociated; and, interestingly, ES cells carrying a targeted mutation in both *c-jun* alleles cannot contribute to the hepatic lineage²².

TABLE 1 Viability of wild-type and SF/HGF mutant embryos

Age	Number of litters	Number of pups	Genotype of live embryos		
			+/+	+/-	-/-
E10-12.5	11	101	26	51	24
E14.5	38	295	93	167	35
E16.5	9	55	19	35	1
E18.5	7	45	16	29	0
W3	35	236	85	151	0

Analysis of SF/HGF genotypes in offspring generated from heterozygous intercrosses. The genotypes were identified by PCR analysis on DNA isolated from embryos between days 10 and 18.5 (E10-E18.5) and 3-week-old (W3) mice. In addition to the live embryos, many embryos without heartbeat or with signs of resorption were observed, the majority of which had a SF/HGF^{-/-} genotype.

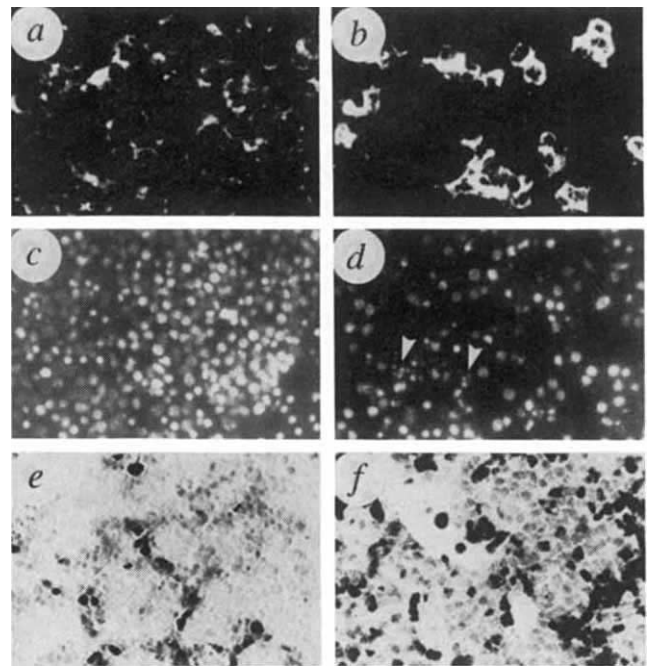


FIG. 3 Histochemical analysis of livers from embryos mutant for the SF/HGF gene. a, b, Cytokeratin immunohistochemistry of livers from wild-type (a) and SF/HGF^{-/-} (b) E14.5 embryos ($\times 300$). c, d, Nuclear morphology of livers from wild-type (c) and SF/HGF^{-/-} (d) E14.5 embryos stained with the dye Hoechst 33258 ($\times 220$). e, f, Haematopoietic islands in livers in wild-type (e) and SF/HGF^{-/-} (f) E14.5 embryos detected by benzidine histochemistry ($\times 220$).

METHODS. Cytokeratin immunohistochemistry was performed on frozen sections with guinea-pig anti-mouse cytokeratin (antibody K11)²⁸ and Cy3-coupled anti-guinea-pig IgG (Dianova). Staining with Hoechst 33258 and the benzidine histochemistry were done on 8- μ m sections from Technovit-7100-embedded livers.

SF/HGF and c-Met have been implicated in formation of tubular epithelia¹¹, early development of the nervous system¹², and angiogenesis^{13,14}. However, the morphological appearance of tubular epithelia in the internal organs of SF/HGF^{-/-} embryos is normal, and so is the development of the lung and kidney in organ culture. The morphological appearance of endothelia or the expression of the endothelial specific marker von Willebrand factor, as analysed by immunohistochemistry, was not altered in the SF/HGF^{-/-} embryos (data not shown). Similarly, no changes were apparent in the developing cardiac and nervous systems. In contrast, the development of the labyrinth layer of the placenta was affected by the SF/HGF mutation (Fig. 2i-m). The number of trophoblast cells was severely reduced, and the entire labyrinth layer appeared disorganized (Fig. 2l, m). These changes are likely to affect the transfer of nutrients and oxygen from the maternal side to the embryo and might therefore contribute to the death of the SF/HGF^{-/-} embryos.

We could not have predicted the phenotype observed on the basis of the known activities of SF/HGF *in vitro* and the expression patterns of SF/HGF and c-met throughout embryogenesis. Earlier studies had shown that SF/HGF induces morphogenesis of epithelia cultured in three-dimensional collagen gels¹¹ and that, when applied ectopically, the factor shows neural-inducing¹² or angiogenic^{13,14} activity. By *in situ* hybridization we detect expression of SF/HGF in the embryo from the onset of gastrulation (E. Andermarcher, A. Surani and E.G., unpublished results). During organogenesis, c-met transcripts are found in various epithelia, for example in the lung, kidney or intestine, whereas SF/HGF is expressed in mesenchyme in close vicinity¹⁵. However, throughout the second half of gestation the

expression level of SF/HGF is highest in the liver¹⁵. Whereas SF/HGF is produced in cells of the sinusoidal linings, which are mesenchymal in origin, *c-met* is expressed in hepatocytes. Thus, the phenotype of the SF/HGF mutation is consistent with the high level of expression observed in liver and with a function of the factor and its receptor in mesenchymal-epithelial interactions during development. Interestingly, ectopic expression of SF/HGF in liver parenchymal cells leads to increased organ growth²³. Placenta is also an important source of SF/HGF and the results presented here and in another study²⁹ highlight a role for SF/HGF in placental development.

New growth factors and cytokines are often named after their activity in an *in vitro* assay(s) but later it can become evident that these activities have little bearing on the function of the molecule *in vivo*. SF/HGF is an exception to this rule as one of

the activities used for the characterization of the factor (stimulation of DNA synthesis in primary hepatocyte cultures) hinted at a key physiological role of the factor in liver development. This is also supported by our results on ES cells that carry two targeted alleles of the *c-met* gene: *c-met*^{-/-} ES cells cannot contribute to the liver, but contribute to various other organs. In contrast, *c-met*^{+/-} ES cells participate in the development of the hepatic lineage (F.B. and C.B., manuscript in preparation). The death *in utero* of the SF/HGF^{-/-} embryos has prevented us from investigating additional and perhaps equally critical functions of the factor in other processes, such as terminal differentiation or renewal of epithelia, wound healing or cancer spreading. Analysis of the developmental potential of *c-met*^{-/-} ES cells or gene ablation in a tissue-specific fashion²⁴ may provide a strategy for investigating additional roles of SF/HGF. □

Received 6 July 1994; accepted 13 January 1995.

1. Stoker, M., Gherardi, E., Perryman, M. & Gray, J. *Nature* **327**, 239–242 (1987).
2. Gherardi, E. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5844–5848 (1989).
3. Weidner, M. K., Behrens, J., Vandeckerckhove, J. & Birchmeier, W. *J. Cell. Biol.* **111**, 2097–2108 (1990).
4. Nakamura, T. et al. *Nature* **342**, 440–443 (1989).
5. Miyazawa, K. et al. *Biochem. biophys. Res. Commun.* **163**, 967–973 (1989).
6. Hartmann, G. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11574–11578 (1992).
7. Lokker, N. et al. *Eur. molec. Biol. Org. J.* **11**, 2503–2510 (1992).
8. Bottaro, D. P. et al. *Science* **251**, 802–804 (1991).
9. Naldini, L. et al. *Eur. molec. Biol. Org. J.* **10**, 2867–2878 (1991).
10. Weidner, M. K., Sachs, M. & Birchmeier, W. *J. Cell Biol.* **121**, 145–154 (1993).
11. Montesano, R., Matsumoto, K., Nakamura, T. & Orci, L. *Cell* **67**, 901–908 (1991).
12. Stern, C. D. et al. *Development* **110**, 1271–1284 (1990).
13. Bussolino, F. et al. *J. Cell Biol.* **119**, 629–641 (1992).
14. Grant, D. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1937–1941 (1993).
15. Sonnenberg, E., Meyer, D., Weidner, K. M. & Birchmeier, C. *J. Cell Biol.* **123**, 223–235 (1993).
16. Birchmeier, C. & Birchmeier, W. *A. Rev. Cell Biol.* **9**, 511–540 (1993).
17. Matsumoto, K. et al. *Biochem. biophys. Res. Commun.* **181**, 691–699 (1991).
18. Clayton, D., Harrelson, A. & Darnell, J. *Molec. cell. Biol.* **5**, 2623–2632 (1985).

19. Mucenski, M. et al. *Cell* **65**, 677–689 (1991).
20. Clarke, A. et al. *Nature* **359**, 328–330 (1992).
21. Lee, E. et al. *Nature* **359**, 288–294 (1992).
22. Hilberg, F., Aguzzi, A., Howells, N. & Wagner, E. *Nature* **365**, 179–181 (1993).
23. Shiota, G., Wang, T., Nakamura, T. & Schmidt, E. *Hepatology* **19**, 962–972 (1994).
24. Gu, H., Marth, J., Mossmann, H. & Rajewsky, K. *Science* **265**, 103–106 (1994).
25. Mansour, S. L., Thomas, K. R. & Capecchi, M. *Nature* **336**, 348–352 (1988).
26. Kuehn, R., Rajewsky, K. & Mueller, W. *Science* **254**, 707–710 (1991).
27. Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (ed. Robertson, E.) 113–157 (IRL, Oxford, 1987).
28. Denk, H., Lackinger, E., Cowin, P. & Franke, W. *Exp Cell Res.* **161**, 161–171 (1985).
29. Uehara, Y. et al. *Nature* **373**, 702–705 (1995).

ACKNOWLEDGEMENTS. C.S. and F.B. contributed equally to this work. We are grateful to K. Rajewsky, W. Müller and R. Kühn for a gift of E14-1 ES cells and advice on blastocyst injections and culture conditions for ES cells. We also thank W. Birchmeier for discussion and reading of the manuscript, A. Smith (MRC Laboratory of Molecular Biology, Cambridge) for the ES cells genomic library, A. Aguzzi and J. Anderson for advice on the pathology of the embryos, W. Franke for anti-mouse cytokeratin antibodies, S. Rieke, A. Rehaus and K. Lane for technical assistance, and U. Ringelsen for help with artwork. This study was supported in part by a grant of the Commission of the European Communities (Human Capital and Mobility Programme) to E.G. and C.B. and by a grant of the Bundesministerium für Forschung und Technologie to C.B.

Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor

Yoshihiko Uehara^{*†}, Osamu Minowa[†],
Chisato Mori[‡], Kohel Shiota[‡], Junko Kuno[†],
Tetsuo Noda[†] & Naomi Kitamura^{*§}

^{*} Institute for Liver Research, Kansai Medical University, Moriguchi, Osaka 570, Japan

[†] Department of Cell Biology, Cancer Institute, Tokyo 170, Japan

[‡] Department of Anatomy, Kyoto University, School of Medicine, Kyoto 606, Japan

HEPATOCYTE growth factor/scatter factor (HGF/SF) functions as a mitogen, motogen and morphogen for a variety of cultured cells^{1–7}. The genes for HGF/SF and its receptor (the *c-met* proto-oncogene product⁸) are expressed in many tissues during the embryonic periods and in the adult^{9–14}. HGF/SF is thought to mediate a signal exchange between the mesenchyme and epithelia during mouse development¹⁵. To examine the physiological role of HGF/SF, we generated mutant mice with a targeted disruption of the HGF/SF gene. Here we report that homozygous mutant embryos have severely impaired placentas with markedly reduced numbers of labyrinthine trophoblast cells, and die before birth. The growth of trophoblast cells was stimulated by HGF/SF *in vitro*, and the HGF/SF activity was released by allantois in primary culture of normal but not mutant embryos. These findings

suggest that HGF/SF is an essential mediator of allantoic mesenchyme-trophoblastic epithelia interaction required for placental organogenesis.

A targeting construct was designed to disrupt the HGF/SF gene by inserting a *neo*^r gene into a partially deleted exon 5 (Fig. 1a). The exon 5 encodes the first kringle domain essential for the biological activities of HGF/SF¹⁶. The construct was introduced into embryonic stem (ES) cells, and three independent clones containing the predicted mutant allele produced chimaeric mice that transmitted the mutation through the germ line (Fig. 1b). Mice heterozygous for the mutation appeared normal and were intercrossed to generate homozygous mutants. None of 61 live-born offspring derived from all three targeted clones were found to be homozygous (Table 1), indicating that the mutants die during the embryonic period. To determine the time of death, pups from days 8.5 to 17.5 of gestation (where plug day is day 0) were genotyped (Fig. 1b, c). The proportion of live homozygous embryos decreased, with the time of death varying between days 13.5 and 15.5 (Table 1).

TABLE 1 Viability of embryos from HGF/SF heterozygous matings

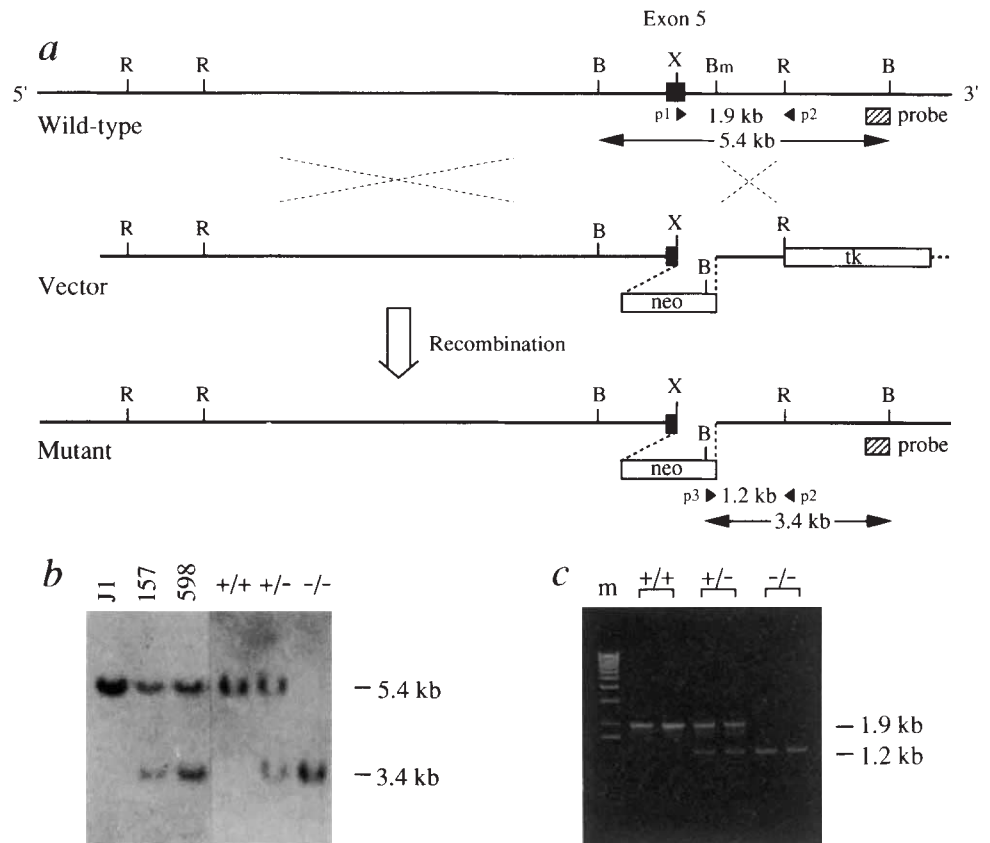
Age*	Number of litters	Genotype of live embryos		
		+/+	+/-	-/-
E8.5–9.5	15	37	64	37
E10.5–11.5	8	24	26	23
E12.5	4	12	16	10
E13.5	5	12	24	8 (1)†
E14.5	4	7	18	3 (8)†
E15.5	3	9	13	3 (4)†
E16.5–17.5	6	11	27	0 (12)†
W3	11	21	40	0

* E8.5 to E17.5, and W3 represent days 8.5 to 17.5 of gestation, and 3 weeks after birth, respectively.

† In parentheses, the numbers of dead homozygous embryos.

§ To whom correspondence should be addressed.

FIG. 1 Targeted disruption of the HGF/SF gene. **a**, Targeting strategy. Wild-type HGF/SF locus (top), targeting vector (middle) and predicted mutant locus (bottom). The targeting vector was created by replacing the *XhoI*-*Bam*HI fragment containing the 3' part of the exon 5 with a *PGK-neo* cassette²⁵. The vector also includes a *PGK-tk* cassette²⁵ at the 3' end of the short homologous segment. Arrowheads represent the positions of PCR primers (p1, 5'-ACCTGGTGTTCACAAAGCAATCCAGAGGT-3'; p2, 5'-CCTATTGCTCATGGGTGATTGAC-3'; p3, 5'-GCCAAGTCTCAATCCATCAGAAG-3'). R, *Eco*RI; B, *Bgl*II; X, *Xho*I; Bm, *Bam*HI. **b**, Southern blot analysis of targeted ES clones and embryos from a heterozygous cross. DNA from parental ES cells (J1), targeted clones (157 and 598), and embryos of wild-type (+/+), heterozygote (+/-) and homozygote (-/-) was digested with *Bgl*III and hybridized to the external probe (~0.6 kb *Ava*I-*Bgl*III fragment). The positions of bands corresponding to the wild-type allele (5.4 kb) and the mutant allele (3.4 kb) are indicated. A *neo* probe confirmed a single integration of the vector in these ES clones (data not shown). **c**, PCR analysis for genotyping embryos. The fragments are generated from the wild-type allele (1.9 kb) and the mutant allele (1.2 kb).



METHODS. Genomic clones of the mouse HGF/SF gene was isolated from a library of strain 129 using a rat HGF/SF cDNA probe²⁶. The targeting vector includes the 11-kb upstream region and the 1.2-kb downstream region of exon 5. J1 ES cells²⁷ were electroporated with the linearized targeting vector at 400 V, 35 μ F, and selected with G418 (0.3 mg ml⁻¹) and FIAU (0.2 μ M) on embryonic fibroblast feeder cells²⁸.

At embryonic day 13.5 (E13.5), 8 of the 9 homozygous mutant embryos were alive, and the live embryos were externally normal apart from their slightly smaller size (Fig. 2a). By contrast, the placentas of the homozygous mutants were obviously different from those of the wild-type or heterozygous embryos: they were paler and smaller (Fig. 2a). RNA protection and *in situ* hybridization analysis have revealed that the HGF/SF gene is expressed in a variety of tissues, including the liver, lung and kidney during mouse development¹⁵. However, histological analysis showed no anomalies in these tissues of E13.5 mutant embryos (Fig. 2b-e). Furthermore, there was no detectable difference in histological structures of other organs, including those of the central nervous and cardiovascular systems, between the wild-type and mutant embryos (Fig. 2f,g and data not shown). Liver deficiency in E14.5 mutant embryos is reported by another group¹⁷. We observed reduced size of the E13.5 mutant liver but could not detect abnormalities in histological structures (Fig. 2b,c). HGF/SF might come to affect liver development distinctly after E14. In contrast, comparison of E13.5 normal placentas with those of the homozygous mutants by histological analysis revealed that the labyrinth region in the mutant placenta was obviously abnormal (Fig. 2h-k). The labyrinth region in the normal placenta has numerous fine embryonic vessels surrounded by trophoblast epithelial cells which are bathed in maternal blood (Fig. 2h,j and ref. 18). In the labyrinth region of the mutant placenta, the number of the trophoblast cells was markedly decreased, resulting in a drastically reduced size of the region. Furthermore, the network of embryonic vessels and maternal sinuses was poorly developed (Fig. 2i,k). The number of spongiotrophoblast cells of the junctional zone was

Seven targeted clones were obtained from 700 doubly resistant colonies. To generate chimaeric mice, these clones were injected into C57BL/6 blastocytes and these blastocytes were implanted into pseudopregnant MCH-ICR females²⁹. Chimaeric males were mated to C57BL/6 females, and heterozygous offspring were intercrossed to produce subsequent generations.

about the same in the mutant and normal placentas. At the early stage of development, nutrients are transported from the mother to embryo through the yolk sac. From around E11 onward, the labyrinth region of the placenta starts to function as a nutrient transport unit¹⁹. Thus, the transport of nutrient to the embryo may be limited in the damaged labyrinth region of the homozygous mutants. This limitation may cause embryonic lethality.

Decrease of the number of trophoblast cells in the mutant labyrinth was first recognized at E10.5 (data not shown), and was apparent at E11.5 (Fig. 2l,m). Although the derivation of the labyrinthine trophoblast cells is not fully understood, these cells are considered to have originated from the extraembryonic ectoderm^{20,21}. Around E8.5, the allantoic mesenchyme comes into contact with the extraembryonic ectoderm, and mitotic activity in the trophoblast cells is low at E8 and markedly increased by E10 (ref. 22). Based on these findings, it has been suggested that the contact of allantoic mesenchyme stimulates the mitotic activity in trophoblast cells. A stimulator(s) produced by allantois may affect the growth of trophoblast cells. Thus, HGF/SF is a candidate for a stimulator produced by allantois. We examined whether allantoic cells produce HGF/SF using an epithelial cell scattering assay⁵, which detects the HGF/SF activity sensitively. The cell scattering activity was detected in the culture medium of allantoic fibroblasts of wild-type (16-32 units per 5×10^4 cells) or heterozygous embryos (4-8 units per 5×10^4 cells), but was below the detectable level in the medium of allantois of homozygotes (Fig. 3a,b). We next examined whether the trophoblast cells are a target for HGF/SF. Analysis of placentas stained with an antibody to the Met receptor showed that the receptor was expressed in the tropho-

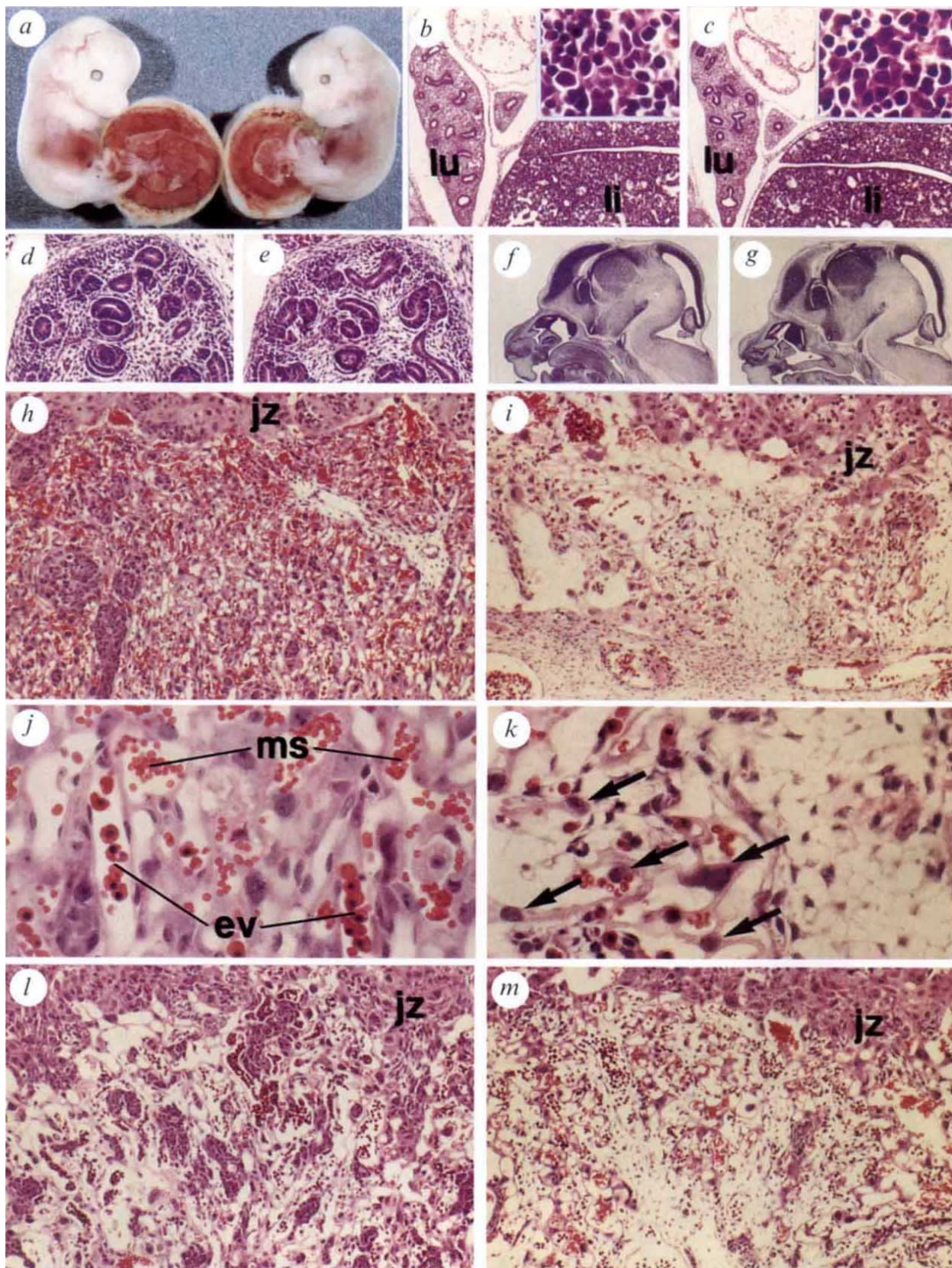
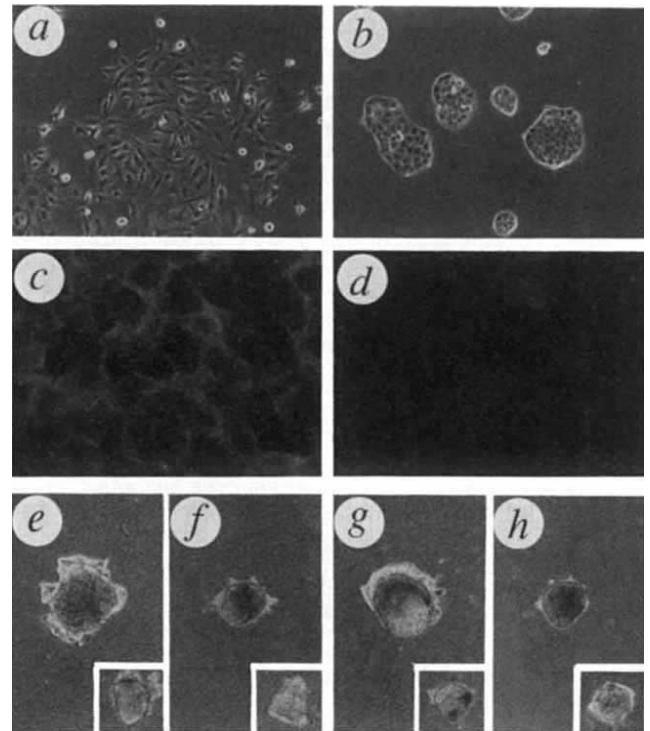


FIG. 2 Morphology of homozygous mutant embryos and placentas. *a*, External appearance at E13.5. Left, wild-type; right, mutant. *b–g*, Sagittal sections through wild-type (*b, d, f*) and mutant (*c, e, g*) E13.5 embryos. *b, c*, Liver (li) and lung (lu) (magnification, $\times 15$). Insets, higher magnification of the liver ($\times 200$). *d, e*, Kidney ($\times 50$). *f, g*, Head ($\times 4$). *h–m*, Sections through wild-type (*h, j, l*) and mutant (*i, k, m*) placentas. *h, i*, The labyrinth region at E13.5 ($\times 50$). *j, k*, Higher-magnification views of *h, i*, respectively ($\times 200$). Trophoblast cells (arrows) are rarely seen

in the mutant labyrinth region, in which fewer embryonic vessels, maternal sinuses and more mesenchymal cells are observed. *l, m*, The labyrinth region at E11.5 ($\times 50$). The number of trophoblast cells is already fewer in the E11.5 mutant labyrinth. jz, Junctional zone; ms, maternal blood sinus; ev, embryonic blood vessel.

METHODS. Embryos and placentas were fixed in 4% paraformaldehyde dehydrated and embedded in paraffin. Sections (5 μ m) were stained with haematoxylin and eosin according to standard procedures.

FIG. 3 HGF/SF activity released by allantois (a, b). Morphology of MDCK cells in the 4-fold-diluted conditioned medium of wild-type allantoic fibroblasts (a), and in the 2-fold diluted conditioned medium of homozygous mutants (b); magnification, $\times 48$. Immunofluorescence staining of trophoblast cells with an anti-Met antibody (c, d). Staining in the absence (c) or presence of a competing peptide (d) ($\times 160$). Growth stimulation of the trophoblast tissue by HGF/SF (e–h). Extraembryonic ectoderm from an E8.5 wild-type placenta (e, f) and an E8.5 mutant placenta (g, h) was cultured for 4 days with (e, g) or without 50 ng ml⁻¹ HGF/SF (f, h). Insets, same tissue after 1 day in culture ($\times 20$). The tissue size roughly reflected the number of cells in the tissue (data not shown). METHODS. For the *in vitro* experiment, E8.5 conceptuses were used in which allantois was not yet in contact with extraembryonic ectoderm. Allantois and extraembryonic ectoderm were dissected from the conceptuses as described²¹. Four to six allantoises were placed in the wells of 96-well plates and cultured in DME medium containing 20% FCS until allantoic fibroblasts grew to confluence. The conditioned medium was obtained by culture of the confluent cells in fresh medium for 3 days ($4\text{--}6 \times 10^4$ cells per 150 μ l). For the scattering assay, the MDCK epithelial cells were incubated in serially diluted conditioned medium for 24 h^{16,30}. The activity unit was defined as in ref. 5. The extra-embryonic ectoderm was divided into two pieces. Each was placed on a collagen gel in DME medium containing 5% FCS, and cultured in the presence or absence of recombinant human HGF/SF. For immunohistology, E10.5 placentas were dissected and fixed in 4% paraformaldehyde. The fixed tissues were equilibrated with 20% sucrose, frozen and sectioned at 5 μ m. Sections were incubated with 1 μ g ml⁻¹ of affinity-purified rabbit polyclonal SP260 antibody¹³ overnight at 4 °C. After washing with PBS, sections were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin diluted at 1:100 for 2 h at room temperature.



blast cells in the labyrinth region (Fig. 3c, d). To examine the effect of HGF/SF on the growth of trophoblast cells, the extra-embryonic ectoderm, which consists of trophoblast cells²¹, was cultured on a collagen gel. Exogenously added HGF/SF stimulated the growth of trophoblast tissue of the wild-type (Fig. 3e, f), as well as the homozygous mutant placenta (Fig. 3g, h), indicating that the trophoblast cells respond to HGF/SF. These results suggest that HGF/SF secreted from allantois functions as a factor that stimulates the growth of trophoblast cells in the labyrinth region. Because HGF/SF is expressed in mesenchymal cells and the receptor is expressed in epithelial cells in close vicinity in various tissues of mouse embryo, it is thought to function as an effector of mesenchymal–epithelial interactions that govern morphogenesis of epithelial cells during the embryonic periods¹⁵. Our findings suggest that HGF/SF is also crucial in interactions required for placental organogenesis.

HGF/SF has several distinct biological functions. It is a potent mitogen for hepatocytes in primary culture as well as other cell lines^{1,2,4}, and stimulates the dissociation and motility of epithelial cells⁵. It also functions as an inducer of epithelial tubulogenesis and of angiogenesis^{7,23,24}. These functions are thought to be required for embryogenesis and tissue remodelling. We have demonstrated here a placental defect and embryonic lethality in mice lacking the HGF/SF gene as direct evidence that HGF/SF is an essential factor for mouse development. □

17. Schmidt, C. et al. *Nature* **373**, 699–702 (1995).
18. Muntener, M. & Hsu, Y.-C. *Acta anat.* **98**, 241–252 (1977).
19. Garbis-Berkvens, J. M. & Peters, P. W. J. in *Pharmacokinetics in Teratogenesis: I* (eds Nau, H. & Scott, W. J. J.) 13–44 (CRC, Boca Raton, 1987).
20. Peel, S. & Bulmer, D. J. *Anat.* **124**, 675–687 (1977).
21. Rossant, J. & Tamura-Lis, W. J. *Embryol. exp. Morph.* **62**, 217–227 (1981).
22. Barlow, P. W. & Sherman, M. I. *J. Embryol. exp. Morphol.* **27**, 447–465 (1972).
23. Bussolino, F. et al. *J. Cell Biol.* **119**, 629–641 (1992).
24. Grant, D. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1937–1941 (1993).
25. Rudnicki, M. A., Braun, T., Hinuma, S. & Jaenisch, R. *Cell* **71**, 383–390 (1992).
26. Okajima, A., Miyazawa, K. & Kitamura, N. *Eur. J. Biochem.* **193**, 375–381 (1990).
27. Li, E., Bestor, T. H. & Jaenisch, R. *Cell* **69**, 915–926 (1992).
28. Takeshima, H. et al. *Nature* **369**, 556–559 (1994).
29. Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (eds Robertson, E. J.) 113–151 (IRL, Oxford, 1987).
30. Uehara, Y. & Kitamura, N. *J. Cell Biol.* **117**, 889–894 (1992).

ACKNOWLEDGEMENTS. We thank G. F. Vande Woude for an anti-Met antibody, K. Jishage, H. Takano and N. Nakamura for technical assistance, and S. Miura, H. Shibata, A. Kurishita for discussion. This work was supported in part by a research grant from the Ministry of Education, Science and Culture of Japan.

Regulation of furrow progression in the *Drosophila* eye by cAMP-dependent protein kinase A

David I. Strutt, Volker Wiersdorff & Marek Mlodzik*

Differentiation Programme, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

THE earliest physical sign of differentiation in the *Drosophila* retina is the passage of the morphogenetic furrow across the epithelium of the eye disc^{1,2}. Secreted factors encoded by *hedgehog* (*hh*)^{3–7} and *decapentaplegic* (*dpp*)^{8–10} have been implicated in propagation of the furrow^{11,12} and the subsequent initiation of photoreceptor differentiation. The morphogenetic furrow initiates

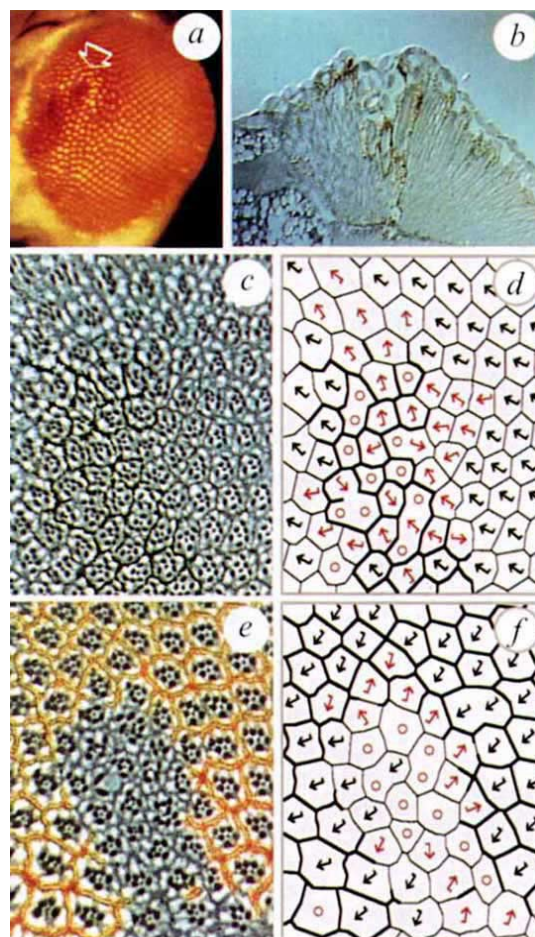
Received 8 July 1994; accepted 13 January 1995.

1. Gohda, E. et al. *J. clin. Invest.* **81**, 414–419 (1988).
2. Zarnegar, R. & Michalopoulos, G. *Cancer Res.* **49**, 3314–3320 (1989).
3. Nakamura, T. et al. *Nature* **342**, 440–443 (1989).
4. Rubin, J. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 415–419 (1991).
5. Stoker, M., Gherardi, E., Perryman, M. & Gray, J. *Nature* **327**, 239–242 (1987).
6. Weidner, K. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 7001–7005 (1991).
7. Montesano, R., Matsumoto, K., Nakamura, T. & Orci, L. *Cell* **67**, 901–908 (1991).
8. Bottaro, D. P. et al. *Science* **251**, 802–804 (1991).
9. Miyazawa, K. et al. *Biochem. biophys. Res. Commun.* **163**, 967–973 (1989).
10. Tashiro, K. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 3200–3204 (1990).
11. DeFrances, M. C., Wolf, H. K., Michalopoulos, G. K. & Zarnegar, R. *Development* **116**, 387–395 (1992).
12. Chan, A. M.-L. et al. *Oncogene* **2**, 593–599 (1988).
13. Iyer, A. et al. *Cell Growth Differ.* **1**, 87–95 (1990).
14. Di Renzo, M. F. et al. *Oncogene* **6**, 1997–2003 (1991).
15. Sonnenberg, E., Meyer, H. K., Weidner, K. M. & Birchmeier, C. *J. Cell Biol.* **123**, 223–235 (1993).
16. Okigaki, M., Komada, M., Uehara, Y., Miyazawa, K. & Kitamura, N. *Biochemistry* **31**, 9555–9561 (1992).

* To whom correspondence should be addressed.

FIG. 1 *pka-C1* homozygous mutant clones in the adult eye cause non-autonomous pattern disruption. Anterior is to the left in all panels. **a**, Non-autonomous external eye roughening caused by a mutant clone of the 89/18 lethal P-element allele of *pka-C1*. Mutant cells (carrying two copies of mutagenic P-element) autonomously express higher levels of pigment. Facets both within and surrounding the clone are disordered, no longer exhibiting the smooth array typical of wild-type tissue. Bulging of the eye surface (arrow) is commonly seen in anteriorly positioned clones, probably because of overgrowth of associated tissue (compare Fig. 3c, f). Clones in the posterior of the eye often appear smooth, as reported previously¹⁵. Mutant clones of an independent P-element allele of *pka-C1*, l(2)01272, consistently gave similar phenotypes. **b**, Section through bulge in *pka-C1*^{89/18} clone shown in **a**, cut in the plane indicated by arrow in **a**. Note the volcano-like appearance with ingression of lens material into eye surface. **c**, Tangential section through *pka-C1*^{89/18} clone. Mutant tissue is marked by higher levels of pigment (visible in pigment cell array surrounding each ommatidium). Note both incorrectly and correctly constructed ommatidia within the clone. **d**, Schematic drawing of clone shown in **c**. Thick lines represent pigment cells of mutant ommatidia, thin lines mark wild-type tissue. The hexagonal array of ommatidia is disrupted both within the clone and in surrounding tissue. Orientation of ommatidia is indicated by arrows, with chirality represented by tails; black indicates normally oriented ommatidia (pointed towards anterior), red indicates abnormal orientation. Circles mark ommatidia with uncertain orientation. Note that almost all ommatidia within the clone exhibit incorrect orientation, and that this defect spreads into surrounding tissue in a non-autonomous manner. **e**, Tangential section through clone of cells mutant for the l(2)01272 P-element allele of *pka-C1*. Mutant tissue is marked by lack of pigmentation. As for *pka-C1*^{89/18} clones, both incorrectly and correctly constructed ommatidia are seen. **f**, Schematic drawing of clone shown in **e**. Thin lines represent pigment cells of mutant ommatidia. As for *pka-C1*^{89/18}, there is non-autonomous disruption of the hexagonal array. Note that whereas wild-type ommatidia are oriented towards the anterior (the normal direction of furrow progression), ommatidia at the edges of the clone point out from the centre of mutant tissue. At the posterior margin of the clone ommatidia are rotated 180° relative to wild-type neighbours.

METHODS. The 89/18 allele was identified in two independent screens from a collection of 2,000 lethal P-element insertions on the second chromosome²⁴; the first screen was for dosage-sensitive interactions with a rough-eye phenotype generated by ectopically expressing *Scabrous* in the furrow (V.W., unpublished results). Second, 600 insertions were screened for visible phenotypes after X-ray-induced mitotic recombination (D.I.S., unpublished results). Complementation analysis showed 89/18 to be allelic to l(2)01272, a lethal P-element insertion at 30C1-2 from the Spradling collection, previously identified as allelic to *pka-C1* (Genomic Center, Berkeley). Reversion of the mutant phenotype by P-element excision was verified for 89/18 (our unpublished



data) and l(2)01272 (Genome Center, Berkeley). Both P elements lie in the first intron of the *pka-C1* gene²³ (Genome Center, Berkeley). Homozygous mutant clones were generated in first instar larvae by X-ray-induced mitotic recombination. *pka-C1*^{89/18} clones were identified by increased pigment levels from the *white*⁺ mini-gene contained by the mutagenic P-element. *pka-C1*⁰¹²⁷² clones were marked by lack of pigment from a *white*⁺-containing P-element carried on the homologous chromosome at 39E. Standard histological methods were used².

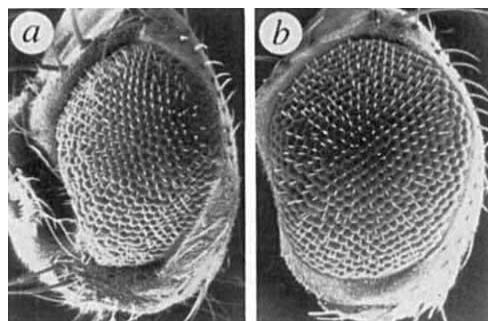
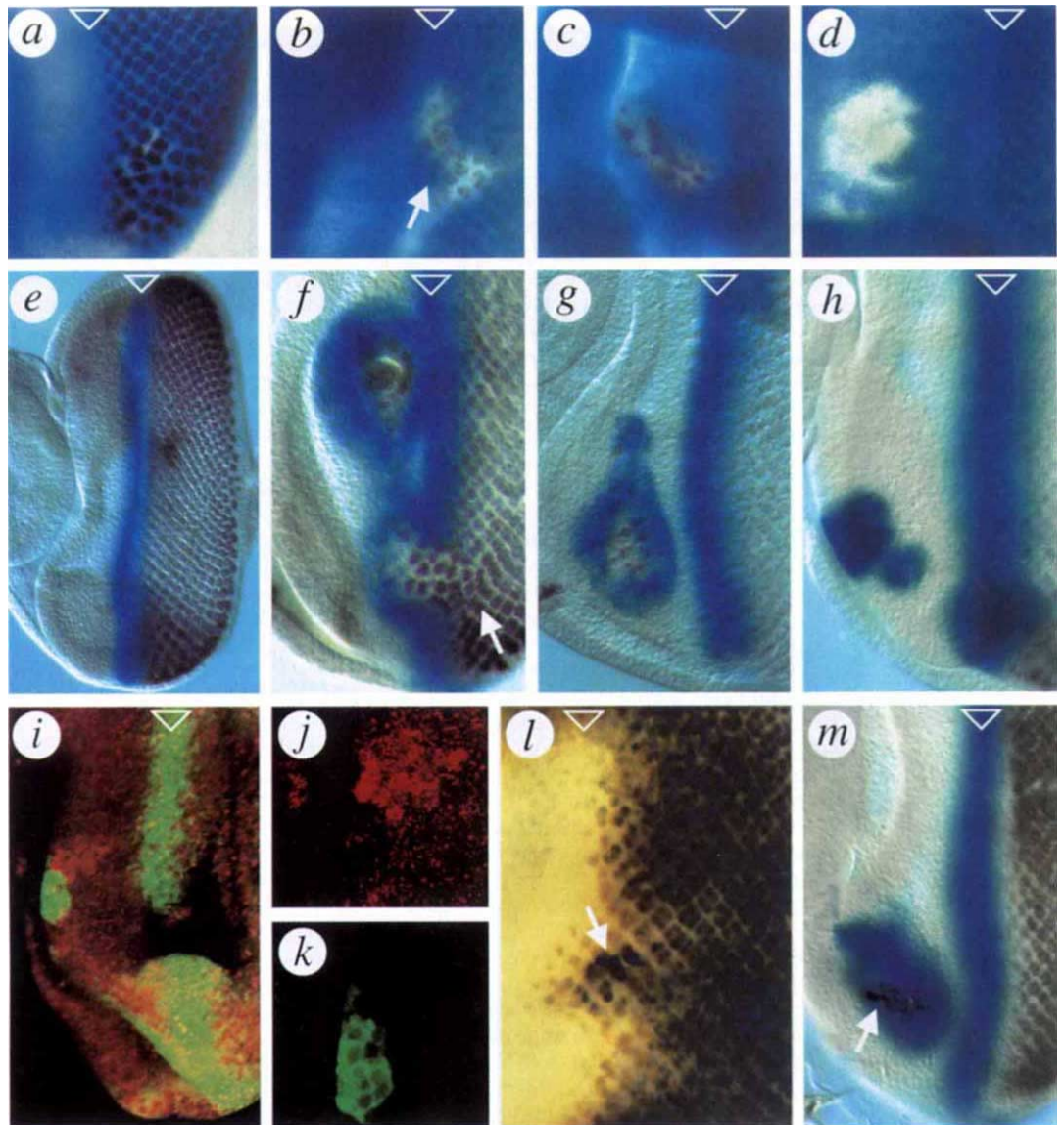


FIG. 2 Dominant suppression of the *hh*^{ts2} phenotype by *pka-C1*. **a**, Scanning electron micrograph of the adult eye of a female homozygous for the *hh*^{ts2} mutation¹² grown at the permissive temperature. Note the reduced size of the eye and disorder of the facets, particularly at the anterior edge (to the left). **b**, Scanning electron micrograph of the adult eye of a female sibling of the fly shown in **a**, homozygous for the *hh*^{ts2} mutation, and carrying one copy of the *pka-C1*^{89/18} lethal P-element insertion. Although the eye still exhibits some disorder, there is an increase in size and less severe disruption, particularly at the anterior edge (to the left). This dominant suppression of the *hh*^{ts2} phenotype by *pka-C1* is similar to the suppression of *hh*^{ts2} shown by mutations in the *ptc* gene¹².

METHODS. Flies of genotype *w*; +/+; *hh*^{ts2} *e*^s/TM6b were crossed to flies of genotype *w*; *pka-C1*^{89/18}/CyO; +/+ at 25 °C; progeny of genotype *w*; *pka-C1*^{89/18}/+; *hh*^{ts2} *e*^s/+ were collected and crossed back to *w*; +/+; *hh*^{ts2} *e*^s/TM6b at the permissive temperature of 18 °C, to obtain progeny of genotype *w*; +/+; *hh*^{ts2} *e*^s/*hh*^{ts2} *e*^s and *w*; *pka-C1*^{89/18}/+; *hh*^{ts2} *e*^s/*hh*^{ts2} *e*^s, which could be positively identified by the combination of markers they carry. Siblings of the same sex were compared in random pairs, and clear suppression was seen in 70% of male pairs (*n*=23) and 61% of female pairs (*n*=56). For scanning electron microscopy, the eyes were prepared by critical-point drying and coated with 2 nm of gold.

FIG. 3 Analysis of *pka-C1* clones in third instar eye imaginal discs. All panels except e and m show discs containing mutant clones for the *pka-C1*^{89/18} allele; m, a disc in which ectopic Hh expression has been induced⁷ (all discs are oriented anterior to the left with the furrow marked by an open arrowhead); e, whole eye disc. All other panels are enlargements of part of the eye disc. a–d, Clones marked by the lack of expression from an *arm-lacZ* reporter²⁵ (blue), and stained to show neuronal differentiation using the anti-ELAV monoclonal antibody (brown). a, Disc containing clone posterior to the furrow at the time of fixation (unstained white tissue). Photoreceptor cluster arrangement is irregular both within and surrounding the clone (compare b, f). But clones very close to the posterior edge of the disc show no defect (data not shown), consistent with their phenotype in adult eyes (Fig. 1a legend). This is probably due to their proximity to the edge of the disc where furrow movement initiates and *dpp* is already expressed¹². b, Clone intersecting the furrow. Photoreceptor clusters expand anterior to the furrow, both within and beyond the confines of mutant area (arrow). Clusters within the clone are more mature than those in surrounding tissue. c, Clone anterior to the furrow. Photoreceptor clusters associated with mutant tissue are separated from clusters behind the advancing furrow. The clone is in a different plane of focus from the rest of the disc, apparently a result of excess cell proliferation causing a bulge (compare Fig. 1a, b). d, Clone more anterior to the advancing furrow. No neuronal differentiation is present, although such clones are often associated with bulges in the disc epithelium, suggesting a stimulation of cell proliferation which is separable from the adoption of neural cell fate. e–h, Discs stained for *dpp-lacZ* reporter¹⁷ expression (blue), again double-labelled for ELAV (brown). e, Disc showing wild-type organization. Only cells in the furrow express *dpp*^{16,17}, whereas neuronal differentiation is seen posterior to the furrow. More mature photoreceptor clusters are in posterior positions, with immature clusters emerging from the furrow. f, Disc with clone showing ectopic neuronal differentiation. Two separate areas of ectopic differentiation are seen, presumably produced by a large clone that separated into two patches during proliferation. Note ectopic *dpp* expression surrounding ectopic photoreceptors. As *dpp* staining lies outside the region of photoreceptor differentiation, we deduce that it must also lie outside the mutant tissue (compare marked clones in b and c). Again ectopic differentiation is associated with a bulge in the disc, placing some ELAV-positive clusters out of plane of focus. Arrow marks the area of confrontation between photoreceptor clusters of ectopic origin, and those from the normal furrow. Note the spread of disorder produced by the resulting 'crowding', which we interpret as a consequence of the intersection of an ectopic and an endogenous furrow. g, Disc showing ectopic neuronal differentiation in a region clearly separated from the furrow, again surrounded by ectopic *dpp* expression. h, Disc showing ectopic *dpp* expression, but no ectopic neuronal differentiation. Note that *dpp* expression is not in a ring, but in patches (compare d). Isolated anterior patches of *dpp* staining are often associated with bulges in the epithelium, suggesting increased cell proliferation, consistent with the effect of *dpp* expression in other imaginal discs^{8,26,27}. i, Double immunofluorescence image of disc containing *pka-C1* clone, showing expression of the Myc-epitope clonal marker (red) and *dpp-lacZ* reporter expression (green). A patch of ectopic *dpp* expression is seen in the anterior of the disc (compare h). j, Confocal section of disc shown in i, enlarged for region of *pka-C1* clone, marked by absence of Myc-epitope expression. Nuclear Myc expression is seen in all cells except those in the clone. Note increased Myc in nuclei of the 'twinspot'. Speckled staining present throughout the sample (over both nuclei and cytoplasm) is nonspecific background from the anti-Myc antibody. k, Same confocal section as in j, showing high-level cell-autonomous expression of *dpp* in all mutant cells. l, Disc containing clone double-



stained for ELAV expression (brown) and *hh* expression (grey). The most immature photoreceptor clusters express only ELAV, whereas low levels of *hh* expression are seen 1–2 rows more posteriorly, and high levels are present in more mature clusters^{4,11,12}. A small group of ectopic photoreceptor clusters expressing ELAV is visible intersecting the furrow (compare marked clone in b). The most mature clusters in the centre show *hh* expression (arrow). In 6,000 disc complexes analysed, no cases of ectopic *hh* expression were seen, except in ectopic mature photoreceptor clusters. We conclude that loss of *Pka-C1* activity does not directly induce *hh* expression, other than as a consequence of the photoreceptor differentiation following ectopic induction of *dpp* expression. m, Disc from fly in which ectopic Hh expression has been induced using the *Tub > y+ > hh* transgene⁷, double-stained for ELAV (brown) and *dpp*-expression (blue). A patch of ectopic *dpp* expression is seen anterior to the furrow, within which ectopic photoreceptor differentiation is just beginning as assayed by ELAV expression (arrow). Thus ectopic Hh expression can induce *dpp* expression, and ectopic photoreceptor differentiation, in a similar manner to *pka-C1* clones. For a more complete analysis of the phenotype caused by ectopic Hh expression, see ref. 22. METHODS. Homozygous clones for the *pka-C1*^{89/18} allele were generated as described in Fig. 1. For clones in a–d, larvae were transheterozygous for *pka-C1*^{89/18} and an *arm-lacZ* reporter P-element²⁵ at 28 Å. For experiments in e–h and i–k, the *arm-lacZ* reporter was replaced by a *dpp-lacZ* reporter¹⁷, and for experiment in l, by an *hh* enhancer trap insertion⁴. P[mini-w⁺, hs- π M]²⁸ insertions on 2L were used to mark the clone shown in i–k. Ectopic Hh expression was produced using the *Tub > y+ > hh* transgene⁷ in larvae carrying the *dpp-lacZ* reporter¹⁷. Disc complexes were dissected from wandering third-instar larvae. For experiments in a–h and m, discs were briefly fixed with 0.15% glutaraldehyde before X-Gal staining, and then post-fixed and stained by standard methods² using rat monoclonal anti-ELAV (gift from G. Rubin) and a horseradish peroxidase-conjugated secondary. For i to l, a rabbit polyclonal anti- β -gal antibody (Cappel) with either a FITC-conjugated or HRP-conjugated secondary, and an anti-Myc monoclonal antibody²⁸ with a Texas red-conjugated secondary were used. Pictures were taken on a Zeiss Axiophot using either bright-field or Nomarski optics, except in i to k, for which the EMBL compact confocal microscope was used. Statistical analysis of clone frequency and position within the disc is available on request.

at the posterior edge of the third larval instar eye imaginal disc. Its continued progression towards the anterior is believed to depend upon secretion of Hh protein by the differentiating clusters of photoreceptors that emerge posterior to the moving furrow^{11,12}. This progression is marked by the initiation of expression of the transforming growth factor- β homologue Dpp in cells entering the furrow anteriorly, and loss of *dpp* expression in cells emerging posteriorly^{16,17}. Although the transmembrane protein encoded by the *patched* gene has been genetically implicated as the Hh receptor^{12,18–20}, the intercellular signalling pathways involved in these inductive processes remain uncharacterized. Here we show that the catalytic subunit of cyclic AMP-dependent protein kinase A (Pka-C1)^{13–15} is required for the correct spatial regulation of *dpp* expression during eye development. Loss of Pka-C1 function is sufficient to produce an ectopic morphogenetic wave marked by premature ectopic photoreceptor differentiation and non-autonomous propagation of *dpp* expression. Our results indicate that Pka-C1 lies in a signalling pathway that controls the orderly temporal progression of differentiation across the eye imaginal disc.

During screens for genes involved in early eye development, we identified lethal P-element insertions in the *pka-C1* gene that are capable of disrupting the orderly structure of the adult eye in a non-autonomous manner. Clones of homozygous mutant cells often produce disorder of the ommatidial array which spreads into surrounding wild-type tissue (Fig. 1). In particular, ommatidia surrounding mutant clones are frequently incorrectly oriented relative to their neighbours. A *pka-C1* mutation can suppress the eye phenotype of a weak *hh* mutation¹² (Fig. 2), providing genetic evidence that *pka-C1* and *hh* might be acting in the same pathway during eye development, and hence Pka-C1 might regulate furrow progression. To understand the origin of the adult defects shown by *pka-C1* clones, we analysed their phenotype in third-instar eye discs during furrow progression by staining both for a clonal marker (labelling all non-mutant cells) and for a neuronal marker which labels cells that have adopted the photoreceptor cell fate. Four classes of clones were obtained. Those inspected after the furrow has progressed more anteriorly show no phenotype if they lie right at the posterior edge of the disc, but otherwise exhibit irregular arrangement of photoreceptor clusters (Fig. 3a). Clones in the region of or anterior to the furrow at the time of staining show ectopic differentiation of photoreceptor clusters within and beyond the mutant tissue (Fig. 3b, c). Typically they contain the most mature clusters in the centre. When the progressing furrow is already in contact with the mutant tissue, the ectopic photoreceptors appear as an anterior continuation of the existing photoreceptor field (Fig. 3b). In clones that are still physically separated from the advancing furrow, the ectopic photoreceptors form an island of differentiating cells (Fig. 3c). The fourth class are clones positioned well anteriorly of the furrow, which do not show ectopic expression of the neuronal marker (Fig. 3d).

During eye disc morphogenesis, passage of the furrow and associated *dpp* expression precedes photoreceptor differentiation, which in turn leads to further propagation of the furrow and *dpp* expression, apparently induced by the photoreceptors secreting Hh protein^{11,12,16,17}. To test whether this sequence of events might hold true with regard to the ectopic photoreceptor clusters observed in *pka-C1* mutant clones, we induced such clones in animals carrying a *dpp-lacZ* reporter¹⁷. Ectopic photoreceptor clusters are indeed associated with ectopic *dpp* expression (Fig. 3f, g) and, in particular, dramatic rings surround isolated islands of photoreceptor differentiation located anterior to the advancing furrow. In experiments with marked clones, ectopic neuronal differentiation (when present) is always up to and beyond the clone boundary, so we deduce that this ectopic *dpp* must be non-autonomously induced outside the clone.

Ectopic *dpp* expression was also seen in the absence of ectopic photoreceptor differentiation, but in more continuous patches, and always well anteriorly of the furrow (Fig. 3h). Marked clones were generated to confirm the relationship of *dpp* expres-

sion to the boundaries of clones in the anterior part of the eye disc (Fig. 3i–k), showing that *pka-C1* mutant cells autonomously express *dpp*. Thus, Pka-C1 function is required for the repression of *dpp* expression. As we never observe ectopic *dpp* posterior to the furrow, despite being able to recover *pka-C1* clones in such positions, we conclude that Pka-C1 is required for *dpp* repression only in undifferentiated cells anterior to the furrow.

In the anterior compartment of wing and leg imaginal discs, ectopic expression of the Hh protein is sufficient to induce ectopic *dpp* expression⁷. Using the same 'flip-out' technique⁷, we find that ectopic Hh can also induce ectopic *dpp* anterior to the furrow in the eye disc and give rise to ectopic photoreceptor differentiation (Fig. 3m). However, when *pka-C1* clones were induced in animals carrying an *hh* enhancer trap insertion⁴, no evidence of direct induction of ectopic *hh* expression anterior to the furrow was found, except as a result of ectopic photoreceptor differentiation (Fig. 3l). Thus, there are two contrasting observations: *pka-C1* clones exhibit either ectopic photoreceptor differentiation and associated non-autonomous induction of *dpp* expression (when lying close to the advancing furrow), or show no ectopic photoreceptor differentiation and autonomous *dpp* expression (when lying further anterior). These can be reconciled by the following model: initially all Pka-C1 mutant cells anterior to the furrow express ectopic *dpp*. When such a patch of cells comes to lie closer to the advancing furrow, differentiation is triggered, by the same mechanism as that which acts on *dpp*-expressing cells in the furrow itself. Differentiating photoreceptors within the clone then cease to express *dpp*, but instead induce an ectopic morphogenetic furrow (as revealed by non-autonomous induction of *dpp* expression), presumably by secretion of Hh. The ectopic furrow then propagates beyond the clone, leading to the observed differentiation of photoreceptors outside the limits of *pka-C1* clones, and accounting for the observed non-autonomy of the phenotype. Interestingly, the ectopic furrow propagates in all directions, and is not limited to the anteroposterior axis.

Experiments with transplanted eye discs²¹ have been interpreted to indicate the presence of a 'zone of competence' ahead of the moving furrow, which is able to sustain furrow initiation¹². This is consistent with our observations that *pka-C1* clones are unable to induce photoreceptor differentiation until the endogenous furrow is relatively close. We conclude that within the zone of competence, loss of Pka-C1 activity is necessary and sufficient to induce differentiation and an ectopic morphogenetic wave.

Both loss of Pka-C1 function and ectopic Hh expression can induce *dpp* expression and formation of an ectopic furrow (Fig. 3 and ref. 22). As the induction of *dpp* expression is cell-autonomous in *pka-C1* clones, and as we do not see direct induction of *hh* expression in such clones, we believe that Pka-C1 does not act through *hh*, but probably acts downstream or in a parallel pathway. This view of Pka-C1 action is supported by studies in the developing wing and leg imaginal discs²³. However, the similarity of the phenotypes for *pka-C1* clones and ectopic Hh expression, and the genetic interaction between *pka-C1* and *hh*, leads us to favour the hypothesis that *hh* functions to activate *dpp* by causing inhibition of Pka-C1 expression or activity. □

Received 19 December 1994; accepted 2 February 1995.

1. Ready, D. F., Hanson, T. E. & Benzer, S. *Devl Biol.* **53**, 217–240 (1976).
2. Tomlinson, A. & Ready, D. F. *Devl Biol.* **120**, 366–376 (1987).
3. Nüsslein-Volhard, C. & Weischaus, E. *Nature* **287**, 795–801 (1980).
4. Lee, J. J., von Kessler, D. P., Parks, S. & Beachy, P. A. *Cell* **71**, 33–50 (1992).
5. Lee, J. J. et al. *Science* **266**, 1528–1537 (1994).
6. Tabata, T. & Kornberg, T. B. *Cell* **76**, 89–102 (1994).
7. Basler, K. & Struhl, G. *Nature* **368**, 208–214 (1994).
8. Spencer, F. A., Hoffman, F. M. & Gelbart, W. M. *Cell* **28**, 451–461 (1982).
9. Padgett, R. W., St Johnston, R. D. & Gelbart, W. M. *Nature* **325**, 81–84 (1987).
10. St Johnston, R. D. et al. *Genes Dev.* **4**, 1114–1127 (1990).
11. Heberlein, U., Wolff, T. & Rubin, G. M. *Cell* **75**, 913–926 (1993).
12. Ma, C., Zhou, Y., Beachy, P. A. & Moses, K. *Cell* **75**, 927–938 (1993).
13. Foster, J. L., Higgins, G. C. & Jackson, F. R. *J. biol. Chem.* **263**, 1676–1681 (1988).
14. Kalderon, D. & Rubin, G. M. *Genes Dev.* **2**, 1539–1556 (1988).

15. Lane, M. E. & Kalderon, D. *Genes Dev.* **7**, 1229–1243 (1993).
16. Masucci, J. D., Miltenberger, R. J. & Hoffman, F. M. *Genes Dev.* **4**, 2011–2023 (1990).
17. Blackman, R. K., Sanicola, M., Rafferty, L. A., Gillevet, T. & Gelbart, W. M. *Development* **111**, 657–666 (1991).
18. Hooper, J. E. & Scott, M. P. *Cell* **59**, 751–765 (1989).
19. Nakano, Y. *et al.* *Nature* **341**, 508–513 (1989).
20. Ingham, P. W., Taylor, A. M. & Nakano, Y. *Nature* **353**, 184–187 (1991).
21. Lebovitz, R. M. & Ready, D. F. *Dev. Biol.* **117**, 663–671 (1986).
22. Heberlein, U., Singh, C. M., Luk, A. Y. & Donahue, T. J. *Nature* **373**, 709–711 (1995).
23. Lepage, T., Cohen, S. M., Diaz-Benjumea, F. J. & Parkhurst, S. M. *Nature* **373**, 711–715 (1995).
24. Török, T., Tick, G., Alvarado, M. & Kiss, I. *Genetics* **135**, 71–80 (1993).
25. Vincent, J.-P., Girdham, C. H. & O'Farrell, P. H. *Dev. Biol.* **164**, 328–331 (1994).
26. Diaz-Benjumea, F. J., Cohen, B. & Cohen, S. M. *Nature* **372**, 175–179 (1994).
27. Capdevila, J. & Guerrero, I. *EMBO J.* **13**, 4459–4468 (1994).
28. Xu, T. & Rubin, G. M. *Development* **117**, 1223–1237 (1993).

ACKNOWLEDGEMENTS. For fly stocks, we particularly thank J. P. Vincent and also K. Moses, T. Laverty, K. Matthews and S. Cohen. We thank I. Kiss for allowing us to screen his collection of P-element lethals; U. Heberlein, D. Kalderon, S. Cohen and K. Moses for discussion and for sharing data before publication; S. Cohen and L. Zwiebel for comments on the manuscript; S. Cohen and K. Hartung for assistance with confocal and scanning electron microscopy, respectively. D.I.S. was supported by a Wellcome Trust Travelling Research Fellowship. The first two authors contributed equally to this work.

Growth and differentiation in the *Drosophila* eye coordinated by *hedgehog*

Ulrike Heberlein^{*†}, Carol M. Singh^{*}, Alvin Y. Luk^{*} & Terrence J. Donohoe^{*}

^{*} Gallo Center Bldg 1, Rm 101, [†] Department of Neurology and Program in Neuroscience, University of California at San Francisco, San Francisco General Hospital, San Francisco, California 94110, USA

DIFFERENTIATION of the *Drosophila* retina is asynchronous: it starts at the posterior margin of the eye imaginal disc and progresses anteriorly over two days. During this time the disc continues to grow, increasing in size by approximately eightfold. An indentation in the epithelium, the morphogenetic furrow¹, marks the front edge of the differentiation wave. Anterior progression of the furrow is thought to be driven by signals emanating from differentiating photoreceptor cells in the posterior eye disc^{2,3}. A good candidate for such a signal is the product of the *hedgehog* (*hh*) gene^{4–7}; it is expressed, and presumably secreted, by differentiating photoreceptors and its function is required for continued furrow movement^{2,3}. Here we show that ectopic expression of *hedgehog* sets in motion ectopic furrows in the anterior eye disc. In addition to changes in cell shape, these ectopic furrows are associated with a tightly orchestrated series of events, including proliferation, cell cycle synchronization and pattern formation, that parallel normal furrow progression. We propose that the morphogenetic furrow coincides with a transient boundary that coordinates growth and differentiation of the eye disc, and that *hedgehog* is necessary and sufficient to propagate this boundary across the epithelium.

The role of *hh* in morphogenetic furrow (MF) progression is thought to involve the induction of the transforming-growth-factor- β (TGF- β) family member decapentaplegic (*dpp*)⁸ in the furrow³. To test this directly we induced *hh* ectopically and assayed its effect on the expression of a *dpp-lacZ* reporter⁹. The 'flip-out' method¹⁰ was used to generate randomly positioned clones of cells that express *hh* constitutively under the control of the *Tubulin- α* promoter¹¹ (*Tub* > *hh*). Cells located anywhere anterior to the MF can be induced to express *dpp-lacZ* (Fig. 1*b*). These cells display the typical changes in shape that define the MF, namely apical-basal shortening and apical surface constriction (Fig. 1*f*). Thus, *hh* is sufficient to induce *dpp* transcription and the ensuing morphological changes, both of which are normally associated with the MF.

Occasionally, rings of *dpp-lacZ*-expressing cells, which surround photoreceptor clusters expressing neural markers (Elav¹²; Fig. 1*b, c*) and photoreceptor-specific genes (*glass*¹³ and *boss*¹⁴; not shown), are observed anterior to the MF in *Tub* > *hh* larvae. Because a ring pattern cannot be achieved by clonal growth, this suggests that cells within the *Tub* > *hh* clone initiated neural differentiation and turned-off *dpp* expression, while inducing surrounding cells to express *dpp-lacZ*. Whereas relatively mature photoreceptor clusters are observed in the centre of the clone, young clusters lie immediately adjacent to the *dpp-lacZ* ring, implying radial progression of differentiation. In a few eye discs an ectopic furrow (yellow arrow in Fig. 1*d*) seems to have advanced perpendicularly to the normal MF, possibly initiating in a *Tub* > *hh* clone positioned near the dorsal edge of the disc. Therefore the direction in which the furrow moves appears to be determined by the position of *hh*-expressing cells and may only be restricted by the location of these cells within the eye primordium. In agreement with this proposal, we find that adult ommatidia located near *Tub* > *hh* clones are oriented incorrectly (Fig. 2*f, g*).

What drives these ectopic furrows beyond the boundaries of *Tub* > *hh* clones? Induction of the endogenous *hh* gene (assayed in *Tub* > *hh* larvae carrying an enhancer trap in *hh*⁵) is observed anterior to the MF (Fig. 1*g*). This allows the ectopic furrow to become independent of *Tub* > *hh* expressing cells and advance by the same mechanisms involved in normal furrow progression. Consistently, *Tub* > *hh* clones alter the normal patterning of surrounding wild-type tissue (Fig. 2*d, h*). Ectopic expression of *patched* (*ptc*), the putative *hh* receptor¹⁵, is also observed in the anterior portion of eye discs from *Tub* > *hh* larvae (Fig. 1*h*). Thus, a genetic cascade similar to that underlying signalling across compartment boundaries in other imaginal discs^{16,17} may operate in the MF of the eye disc.

The expression of two genes encoding basic helix-loop-helix proteins, *atonal*¹⁸ (*ato*) and *hairy*¹⁹ (*h*), parallels furrow progression. *atonal*, which is required for neural development in the retina²⁰, is expressed in a stripe of cells located immediately anterior to the MF; as the furrow passes, expression becomes restricted to presumptive R8 cells²⁰ (Fig. 1*i*). This wild-type expression pattern is reproduced, although in circular fashion, within and surrounding ectopic *dpp-lacZ* rings in *Tub* > *hh* larvae (Fig. 1*j*). Hairy protein is expressed anterior to the MF in a band of cells with a sharp posterior border²¹ that coincides closely with the anterior border of *ato* expression (Fig. 1*k*). While ectopic Hairy is seen partly surrounding *Tub* > *hh* clones (Fig. 1*l*), Hairy expression is repressed immediately adjacent to the *dpp-lacZ* domain (white arrow in Fig. 1*l*) when the latter overlaps with the normal domain of Hairy expression. Thus, ectopic *hh* leads to both induction (long range) and repression (short range) of Hairy expression. *hairy* is thought to function, together with the product of the *extramacrochaetae* gene, by repressing neural differentiation anterior to the MF²². Thus, *hh* has the apparently paradoxical function of inducing both an activator, *ato*, and an inhibitor, *h*, of neural development. The precise spatial and temporal regulation of these genes, both induced by the advancing front, may be needed to ensure the gradual progression of the MF and proper pattern formation.

Regulation of the cell cycle is also tightly associated with MF progression; cells anterior to the MF divide asynchronously, whereas cells in the furrow are arrested in G1 (ref. 23). Cell cycle synchronization is thought to be achieved at two points²⁴: cells in G2 are driven into mitosis by *string* (*stg*)²⁵, while cells in G1 are prevented from re-entering S phase by other factors (such as *roughex*²³). Ectopic expression of *stg* (normally expressed in a stripe anterior to the MF^{23,26}, Fig. 1*n*) is observed surrounding and partially overlapping ectopic *dpp-lacZ* domains (Fig. 1*o*). In addition, increased cell proliferation, often leading to discs (Fig. 1*m, d, h*) and adult eyes (Fig. 2*b, c*) with severely distorted morphology, is observed upon ectopic *hh* expression. Thus, *hh* induces cell-cycle synchronization and arrest in the MF

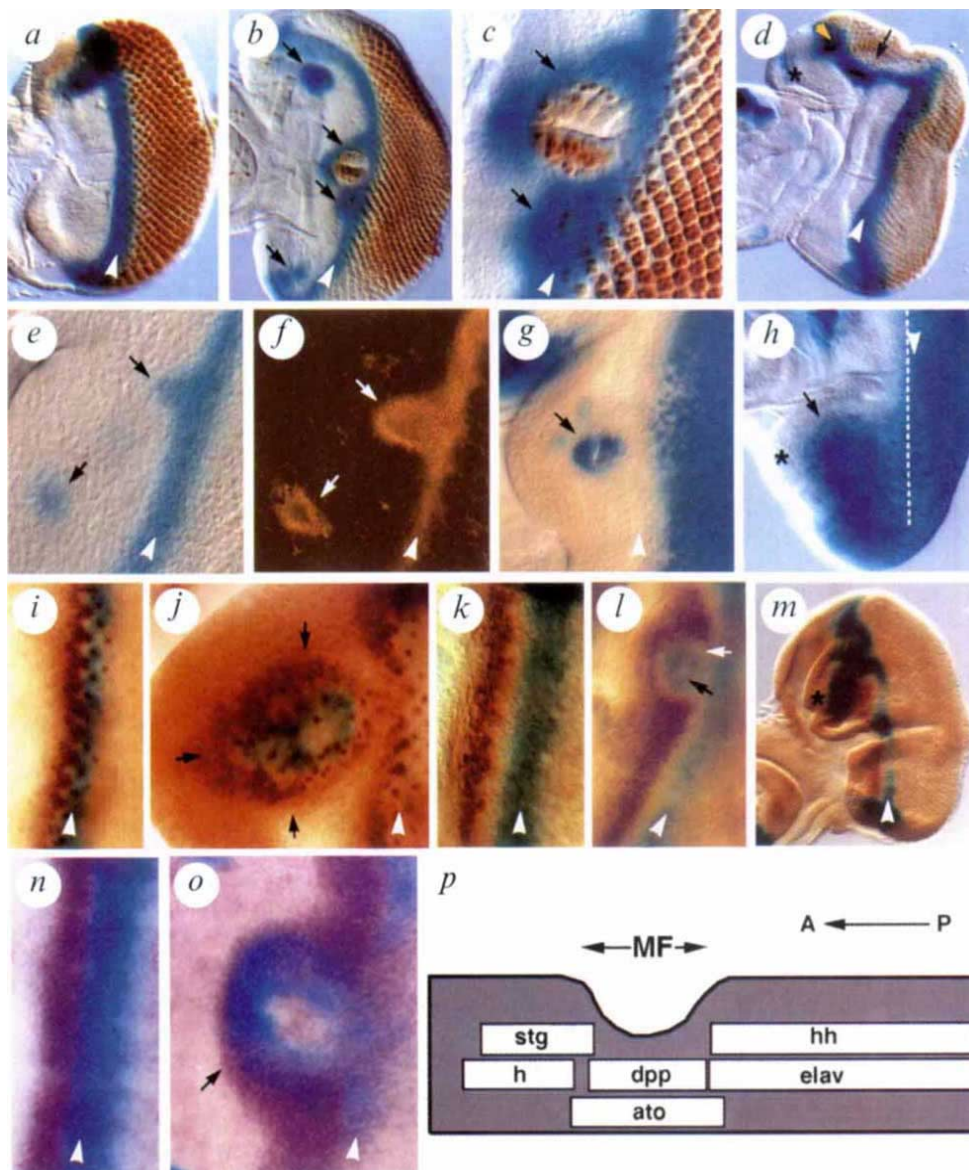
(short range) and cellular proliferation anterior to the furrow (long range).

Whereas ectopic expression of *dpp-lacZ* (and *ptc*) and excessive proliferation can be induced by *Tub > hh* anywhere anterior to the MF, precocious neural differentiation and ectopic expression of *stg* are primarily observed in clones located near the furrow. This suggests the existence of a domain anterior to the MF in which cells are more competent to respond to the *hh*

signal. Although the nature of this 'competence zone' is unknown, it may correspond to the region in which cells have become committed to develop as retina^{2,27}.

In conclusion, we show that *hh* mediates long-range inductive events in the developing eye disc. Whether these effects are completely or only partly mediated by *dpp*, and whether *dpp* and/or *hh* initiate a series of sequential induction or act directly through concentration gradients, remains to be determined. We

FIG. 1 Consequences of ectopic *hh* expression analysed in the eye disc. Clones of cells that constitutively express *hh* under the control of the *Tubulin- α 1* promoter¹¹ (*Tub > hh*) were generated during the late first or second larval instar and consequences were analysed in eye discs dissected from late third-instar larvae. Discs shown in *a-f*, *i-n* and *o* carry a *dpp-lacZ* reporter⁹; discs shown in *g* and *h* carry enhancer trap insertions in *hh*⁵ and *ptc*¹⁷, respectively. Owing to the lack of an appropriate marker (clones are marked by the loss of *yellow*⁺ (*y*⁺) function), *dpp-lacZ* expression was used in double-labelling experiments to identify the general location of *Tub > hh* clones. White arrowheads point to the position of the normal MF. Posterior is to the right. *a-d*, Discs were stained with an antibody against the neural marker *Elav*¹² (brown) and with X-gal (blue). In a normal eye disc (*a*) *dpp-lacZ* is restricted to the MF; differentiating photoreceptors lie posterior to the MF. In eye discs from *Tub > hh* larvae (*b-d*) ectopic *dpp-lacZ* expression (black arrows) is observed anterior to the MF. Ectopic (precocious) neural differentiation can occur in clones located immediately anterior to the MF (*c* shows detail of *b*). Occasionally, an ectopic MF (yellow arrow in *d*) appears to progress orthogonally to the normal MF; excessive cell proliferation (*) precedes the ectopic MF. *e, f*, Images of a *Tub > hh* eye disc stained with X-gal to visualize *dpp-lacZ* (*e*) and rhodamine-phalloidin (*f*). Cells that ectopically express *dpp-lacZ* (black arrows in *e*) display changes in cell shape (small white arrows in *f*) typical of cells in the MF. *g, h*, *Tub > hh* larvae carrying enhancer trap insertions in *hh*⁵ (*g*) and *ptc*¹⁷ (*h*) were stained with X-gal. Ectopic expression of *hh-lacZ* and *ptc-lacZ* (black arrows in *g* and *h*, respectively) is observed anterior to the MF. *hh* is normally expressed in differentiating photoreceptors posterior to the MF^{2,3,5}; *ptc-lacZ* expression normally starts in the MF and extends to the back of the disc (posterior to the white dotted line). Thus, as in the wing disc^{16,17}, *ptc* may repress expression of *dpp* and of *ptc* itself, a repression that is relieved by *hh*. *i, j*, Discs were stained with an antibody against *Ato*²⁰ (brown) and with X-gal (blue). Ectopic expression of *Ato* anterior to the MF (black arrows in *j*) is associated with *Tub > hh* clones. Note that a relatively normal pattern of *Ato* expression (*i*) is reproduced in circular manner surrounding the ectopic *dpp-lacZ* domain (*j*). *k-m*, Discs were stained with an antibody against *Hairy*²¹ (brown) and with X-gal (blue). As seen with *Ato*, ectopic *Hairy* expression partially surrounds the ectopic *dpp-lacZ* domain (black arrow). Note that cells immediately adjacent to *dpp-lacZ*-expressing cells do not express *Hairy* (white arrow in *l*). An example of a disc in which excessive proliferation (*) induced by *Tub > hh* clones led to severely altered morphology (*m*). *n, o*, Discs stained with X-gal (blue) to visualize *dpp-lacZ* were subjected



to *in situ* hybridization²⁸ with a digoxigenin-labelled *stg* probe (purple). A band of *stg* mRNA normally precedes the MF (*n*). In *Tub > hh* larvae ectopic *stg* expression is observed more anteriorly, partly overlapping with and surrounding the ectopic *dpp-lacZ* domain (black arrow in *o*). *p*, The relative domains of expression of genes tested is diagrammed. **METHODS.** Generation of *Tub > hh* clones: *hsFLP1*; *Tub > y*⁺ *hh* males were mated with *hsFLP1*; *dpp-lacZ* (or *hsFLP1*; *ptc-lacZ* or *hsFLP1*; *hh-lacZ*) females. Larval progeny were heat shocked 2–3 times (each for one hour at 38 °C) at 24 h intervals starting 48–72 h after fertilization. Eye discs were dissected from wandering third instar larvae. **Immunohistochemistry:** Fixing and general procedures have been described²⁹. X-gal staining³⁰ was carried out after antibody staining and before phalloidin staining or *in situ* hybridization²⁸. Antibodies: rat anti-*Elav* monoclonal (a gift from G. Rubin), rabbit anti-*Ato*²⁰, mouse anti-*Hairy* monoclonal²¹.

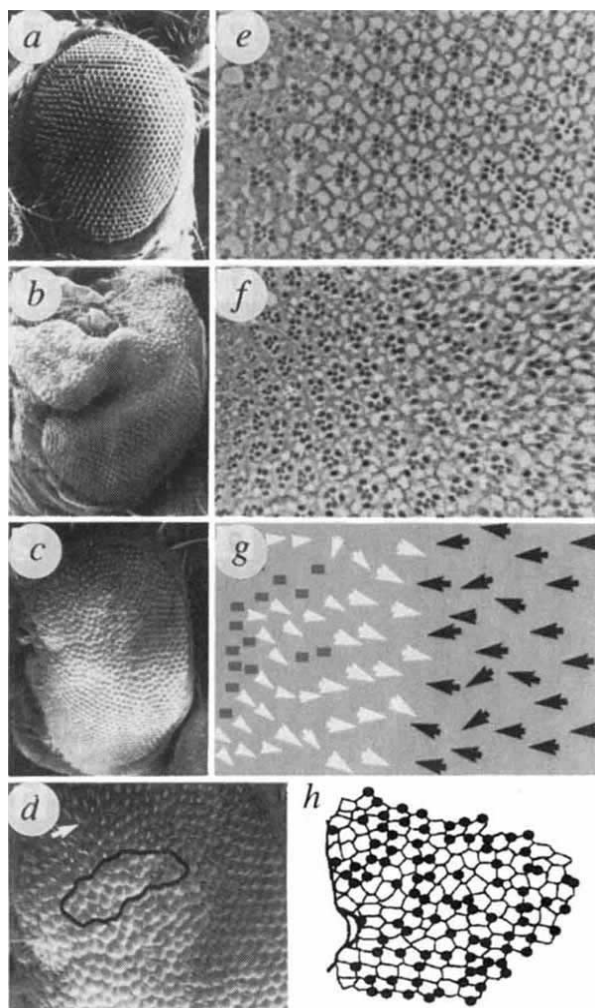


FIG. 2 Consequences of ectopic *hh* expression analysed in the adult eye. Clones were generated as described in the legend to Fig. 1. Posterior is to the right. *a-d*, Scanning electron micrographs of a wild-type eye (*a*) and two eyes (*b*, *c*; *d* is a close-up of *c*) in which *Tub > hh* clones were induced during larval life are shown. Gross overgrowth of the eye is seen in the extreme example shown in *b*. The position of the *Tub > hh* clone is outlined in *d*. Note that the external roughness of the eye spreads into genetically wild-type regions of the eye (white arrow in *d*). *e*, *f*, Tangential sections through a wild-type eye (*e*) and an eye containing a *Tub > hh* clone near the anterior border (*f*). The normal asymmetric array of photoreceptor cells, highlighted by the position of the dark rhabdomeres, is observed in the wild-type eye (*e*). Several rows of ommatidia facing in the opposite direction are observed in a region located posterior to a *Tub > hh* clone in *f*, suggesting that pattern formation occurred in an anterior to posterior direction. *g*, Diagrammatic representation of the orientation (in the anterior/posterior direction) of ommatidia shown in *f*. Ommatidia of aberrant structure (usually observed near the *Tub > hh* clone) and ommatidia whose orientation could not be determined are shown as grey boxes. *h*, Camera lucida representation of a portion of an eye containing a *Tub > hh* clone located near the anterior border. Wild-type (y^+) interommatidial bristles are indicated by filled circles, *Tub > hh* (y^-) bristles are shown as open (grey) circles. In addition to the altered ommatidial arrangement observed in and surrounding the clone, interommatidial bristles are often missing. METHODS. The position of *Tub > hh* clones was determined before sectioning or preparation for SEM by scoring the loss of y^+ function from interommatidial bristles; sections were cut approximately tangentially to the y^- region. External roughness of the eye extended, sometimes substantially, into the genetically wild-type portion of the eye. For the drawing shown in panel *h* the corneas of eyes carrying *Tub > hh* clones (preserved in 100% ethanol) were dissected, mounted in glycerol and photographed at different focal planes. Sections³ and SEM²⁹ were carried out as described.

believe that the role of *hh* in the eye is to coordinate events necessary for proper furrow propagation, such as cell-cycle progression and differentiation. *hh* is not directly required for photoreceptor differentiation^{2,3}; it is, however, required to instruct undifferentiated cells when (and perhaps if) to begin differentiating. Because part of the genetic program set in motion in a cell upon reception of the *hh* signal is to produce *hh* signal, the gradual propagation of the morphogenetic wave across the eye is ensured. □

Received 15 December 1994; accepted 2 February 1995.

1. Ready, D. F., Hanson, T. E. & Benzer, S. *Devl Biol.* **53**, 217–240 (1976).
2. Ma, C., Zhou, Y., Beachy, P. A. & Moses, K. *Cell* **75**, 927–938 (1993).
3. Heberlein, U., Wolff, T. & Rubin, G. M. *Cell* **75**, 913–926 (1993).
4. Mohler, J. & Vani, K. *Development* **115**, 957–971 (1992).
5. Lee, J. J., von Kessler, D. P., Parks, S. & Beachy, P. A. *Cell* **71**, 33–50 (1992).
6. Tabata, T., Eaton, S. & Kornberg, T. B. *Genes Dev.* **6**, 2635–2645 (1992).
7. Tashiro, S. *et al. Gene* **124**, 183–189 (1993).
8. Padgett, R. W., St Johnston, D. & Gelbart, W. M. *Nature* **325**, 81–84 (1987).
9. Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T. & Gelbart, W. M. *Development* **111**, 657–666 (1991).
10. Struhl, G. & Basler, K. *Cell* **72**, 527–540 (1993).
11. Basler, K. & Struhl, G. *Nature* **368**, 208–214 (1994).
12. Robinow, S. & White, K. *Devl Biol.* **126**, 294–303 (1988).
13. Moses, K., Ellis, M. C. & Rubin, G. M. *Nature* **340**, 531–536 (1989).
14. Krämer, H., Cagan, R. L. & Zipursky, S. L. *Nature* **352**, 207–212 (1991).
15. Ingham, P. W. *Curr. Opin. genet. Dev.* **1**, 261–267 (1991).
16. Capdevila, J., Estrada, M. P., Sanchez, H. E. & Guerrero, I. *EMBO J.* **13**, 71–82 (1994).
17. Tabata, T. & Kornberg, T. B. *Cell* **76**, 89–102 (1994).
18. Jarman, A. P., Grau, Y., Jan, L. Y. & Jan, Y. N. *Cell* **73**, 1307–1321 (1993).
19. Carroll, S. B. & Whyte, J. S. *Genes Dev.* **3**, 905–916 (1989).
20. Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. & Jan, Y. N. *Nature* **369**, 398–400 (1994).
21. Brown, N. L., Sattler, C. A., Markey, D. R. & Carroll, S. B. *Development* **113**, 1245–1256 (1991).
22. Brown, N. L., Sattler, C. A., Paddock, S. W. & Carroll, S. B. *Cell* (in the press).
23. Thomas, B. J., Gunning, D. A., Cho, J. & Zipursky, S. L. *Cell* **77**, 1003–1014 (1994).
24. Thomas, B. J. & Zipursky, S. L. *Trends Cell Biol.* **4**, 389–393 (1994).
25. Edgar, B. A. & O'Farrell, P. H. *Cell* **62**, 469–480 (1990).
26. Alphey, L. *et al. Cell* **69**, 977–988 (1992).
27. Lebovitz, R. M. & Ready, D. F. *Devl Biol.* **117**, 663–671 (1986).
28. Tautz, D. & Pfeiffe, C. *Chromosoma* **98**, 81–85 (1989).
29. Kimmel, B. E., Heberlein, U. & Rubin, G. M. *Genes Dev.* **4**, 712–727 (1990).
30. Simon, J. A., Sutton, R. B., Lobell, R. L., Glaser, R. L. & Lis, J. T. *Cell* **40**, 805–817 (1985).

ACKNOWLEDGEMENTS. We thank T. Wolff for help with confocal microscopy, N. Brown and S. Carroll for sharing unpublished information, B. Thomas, G. Mardon, G. Struhl and G. Feger for advice, A. Jarman, Y.-N. Jan, N. Brown and S. Carroll for antibodies, G. Struhl, T. Tabata and R. Blackman for fly stocks, P. O'Farrell for stg DNA, W. Benthall for SEM, J. Triesman, T. Wolff and F. Chanut for critical reading of the manuscript, and G. Rubin, I. Diamond and members of the Gallo Center for generous support.

Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning

Thierry Lepage*, Stephen M. Cohen†‡, Fernando J. Diaz-Benjumea† & Susan M. Parkhurst*

* Division of Basic Sciences, A1-162 Fred Hutchinson Cancer Research Center, Seattle, Washington 98104, USA

† European Molecular Biology Laboratory, Postfach 10.2209, 69012 Heidelberg, Germany

INTERACTION between distinctly specified cells in adjacent compartments establishes organizing centres that control growth and specify cell fate in the developing limbs of *Drosophila*^{1–5}. Localized expression of the secreted Hedgehog protein (Hh) by cells in the posterior compartment^{6–8} induces expression of the secreted signaling molecules *decapentaplegic* (*dpp*) or *wingless* (*wg*) in nearby anterior cells^{2,3}. *wg* and *dpp* in turn organize spatial pattern in the

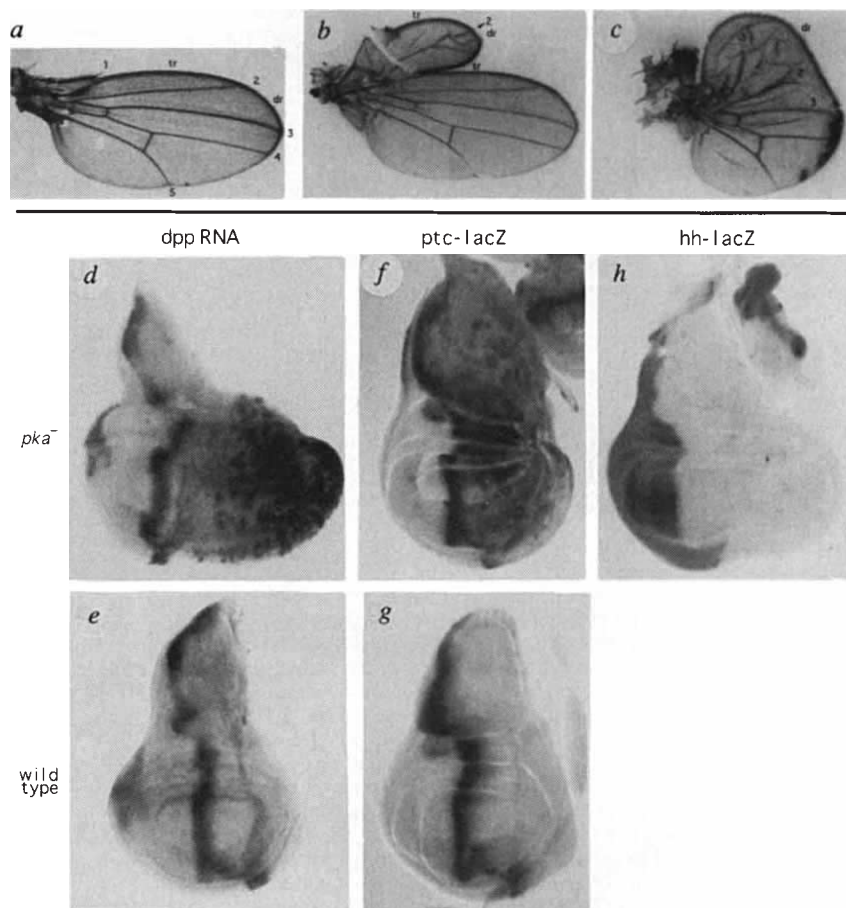
‡ To whom correspondence should be addressed.

wing and leg imaginal discs^{2,4,9}. The Hh signal is thought to act by antagonizing the ability of the *patched* (*ptc*) gene product to repress *wg* and *dpp* expression¹⁰⁻¹⁴. Here we present evidence that removing activity of the gene encoding cyclic AMP-dependent protein kinase A (*pka*) is functionally equivalent to removing *ptc* activity or to providing cells with the Hh signal. These findings suggest that cyclic AMP-dependent protein kinase A is a component of the signal transduction pathway through which Hh and Ptc direct localized expression of *dpp* (or *wg*) and establish the compartment boundary organizer.

Flies homozygous for a viable recessive mutant allele of the gene encoding the catalytic subunit of the cAMP-dependent protein kinase A (*pka-C1*; ref. 15) exhibit pattern abnormalities in the anterior compartments of the wings (Fig. 1a-c). The phenotypes range from subtle deformation of the margin (not shown) to a simple mirror symmetrical duplication of anterior wing elements (Fig. 1b). More complex pattern abnormalities are also

found (Fig. 1c). These phenotypes resemble those produced by viable recessive mutant alleles of *patched* and *costal*, which correlate with ectopic expression of *dpp* in the wing disc (see refs 13, 16, 17 for comparison). Consistent with the phenotypic similarities between *ptc* and *pka* mutants, we observe that *dpp* is ectopically expressed in the anterior compartment of the *pka* mutant discs (Fig. 1d, e). The pattern of *dpp* misexpression in the disc is variable, consistent with the observed variability of the mutant phenotype. The *pka* mutant phenotype also resembles that produced by misexpression of Hh in the anterior compartment of the wing². However, ectopic expression of *hh* is not detectable in *pka* mutant discs (Fig. 1h), suggesting that protein kinase A (PKA) acts downstream in the pathway through which the Hh signal directs localized expression of *dpp*. In addition to repressing *dpp*, Ptc behaves genetically as a repressor of its own expression in the anterior compartment of the wing¹³. We find that a *ptc-lacZ* transgene is ectopically expressed in the anterior

FIG. 1 Duplication of anterior wing pattern in viable mutants of PKA is correlated with ectopic expression of *dpp* and *ptc*, but not *hh*. a, Cuticle preparation of a wild-type wing. The veins serve as positional markers that reflect cell-fate specification along the A/P axis of the wing. The anterior margin of the wing develops a characteristic triple row of sense organs (tr). The first vein is dorsal and meets the wing margin in the proximal part of the wing blade (1), adjacent to the costal region. Vein 2 is ventral and meets the margin near the end of the triple row. Note the transition from tr to a double row of sense organs (dr) in the wing margin between veins 2 and 3. Vein 3 is dorsal and is characterized by campaniform sensillae on the posterior side of the vein (not visible at this magnification). b, Bifurcation of the anterior compartment of the wing from a homozygous *pka*^{P113/2} fly. The supernumerary wing contains two anterior wing margins (tr) and two first veins. The plane of symmetry lies close to a vein 2 (2, arrow). Note the transition to a double row sense organ pattern near the tip of the duplication (dr), indicating that cells at the margin have adopted a fate which normally lies between veins 2 and 3. Bifurcation of the wing and of the haltere is seen in ~20% of homozygotes. About 30% of homozygous flies show a less well developed bulge in the costal region (not shown). Axis bifurcation in the notum and leg defects were observed at lower frequency. c, Reorganization of the anterior compartment of the wing in a fly trans-heterozygous for *pka*^{P113/2} and a deletion that removes the gene. The average severity and penetrance of the phenotype is increased when the viable allele is hemizygous. In this example the tr has been replaced by dr bristles which normally begin between veins 2 and 3. In addition, a large part of the internal wing blade adopted vein 3 identity. There is a supernumerary vein 3, indicated by its dorsal position and the appearance of campaniform sensillae, not visible at this magnification. Ectopic campaniform sensillae are also seen in the intervein region (small arrow). The same range of phenotypes is found in trans-heterozygotes of P113/2 and P01272 or P89/18. d, Ectopic expression of *dpp* in the anterior compartment of the *pka* mutant disc. Note the distortion of the anterior edge of the wing disc, but the pattern of ectopic expression is irregular and varies from disc to disc. *dpp* is not ectopically expressed in the posterior compartment. e, Expression of *dpp* mRNA along the A/P compartment boundary in a wild-type wing disc. f, The *ptc-lacZ* transgene is ectopically expressed in the anterior compartment of the mutant disc. The example shown here exhibits a less severe distortion of the anterior compartment, and presumably would produce a wing with a mild phenotype. g, Expression of a *ptc-lacZ* transgene is highest along the A/P compartment boundary, in the wild-type wing, in the region where *dpp* is expressed. h, The *hh-lacZ* transgene is not misexpressed in the anterior compartment of a severely abnormal *pka*



mutant disc, but is expressed normally in the posterior compartment. METHODS. Three alleles of the *pka-C1* gene were used. P113/2 and P89/18 were isolated from a collection of P-elements on the 2nd chromosome²⁶. 1(2)01272 is a P-element insertional mutant of *pka-C1* (reference: Flybase). In all 3 cases the mutant phenotype has been reverted by excision of the P element (data not shown; P89/18 by V. Wiersdorff and D. Strutt.; 1(2)01272 by the *Drosophila* genome project). Excision of the P113/2 P-element in phenotypic revertants was monitored by PCR amplification of the insertion site. Insertion sites of P113/2, P89/18 and 1(2)01272 were determined by sequencing DNA isolated by plasmid rescue. *pka*^{P113/2} is a P-lacW insertion located 15 bp upstream from the transcription start site defined in ref. 15. 113/2 is a nested insertion of 2 P-elements, one incomplete (data not shown). *pka*^{P89/18} and *pka*^{P01272} are associated with P-element insertions in the first intron. Sequence flanking *pka*^{P89/18} and *pka*^{P01272} are AGCTGTTT and CACTACGGCATCAGCTGT respectively (overlapping sequence is underlined). *dpp* mRNA was visualized by *in situ* hybridization using an antisense RNA probe.

compartment of *pka* mutant wing discs (Fig. 1f, g), suggesting that PKA acts in the signalling pathway by which Ptc represses expression of target genes, including *ptc* and *dpp*.

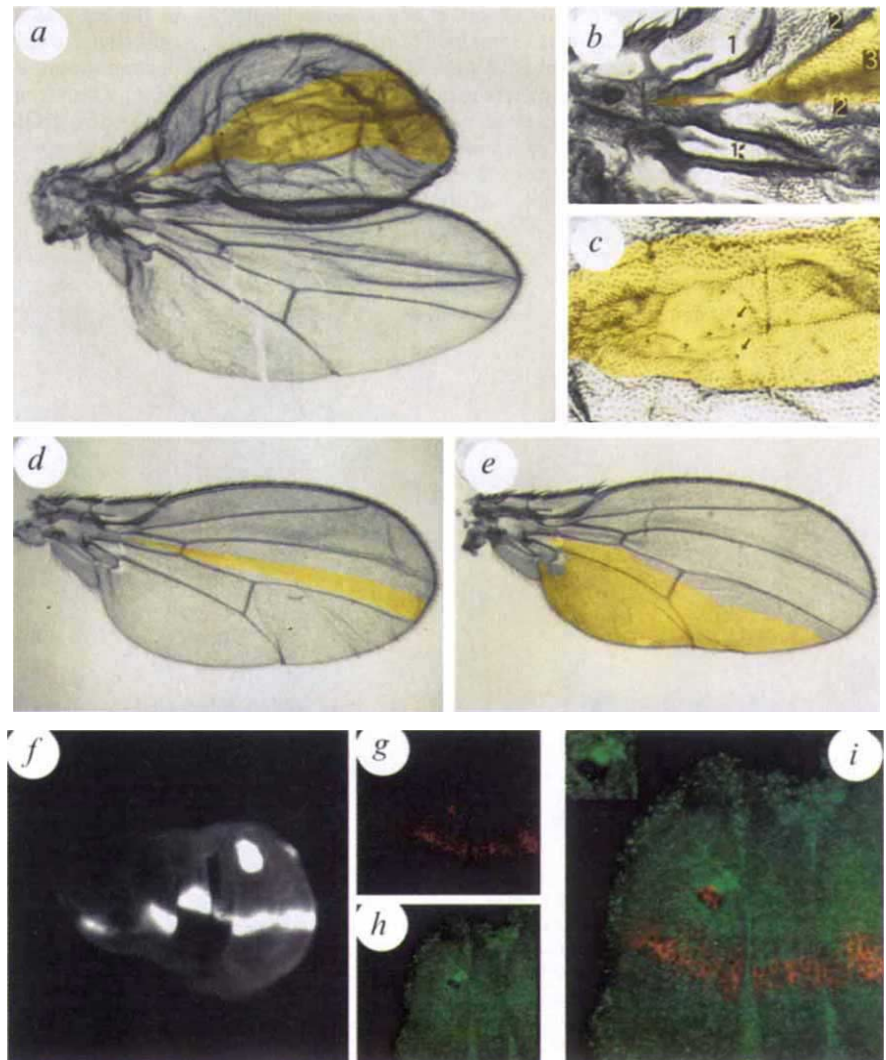
Clones of cells homozygous mutant for a lethal allele of *pka* result in reorganization of the anterior-posterior (A/P) pattern of the wing, producing phenotypes similar to those caused by the viable allele (Fig. 1) or by ectopic expression of Hh (ref. 2). Figure 2a shows a clone of *pka* mutant cells in the anterior compartment which causes a bifurcation of the wing. The clone runs as a continuous strip of cells in the centre of the supernumerary wing, on both the dorsal and ventral surfaces. The clone exerts a non-autonomous influence on the developmental fate of the surrounding wild-type cells, and serves as the axis of symmetry for the flanking duplications (Fig. 2a, b). Note the formation of structures characteristic of vein 3 within the clone

(Fig. 2c), indicating that *pka* mutant cells have adopted the identity of cells normally located close to the A/P compartment boundary.

These observations indicate that clones of *pka* mutant cells serve as the source of a non-autonomous positional signal capable of reorganizing the pattern of the surrounding tissue. Consistent with the observed misexpression of *dpp* in wing discs of the viable allele, clones of *pka* mutant cells serve as a source of ectopic *dpp* expression (Fig. 2f-i). The *pka* mutant cells activate expression of a *dpp-lacZ* transgene reporter in a cell-autonomous manner, suggesting that the PKA signal directs repression of *dpp* transcription. These observations suggest that ectopic expression of *dpp* is the cause of the phenotype produced by the *pka* mutant clones. Consistent with this interpretation, the altered patterns induced by *pka* mutant clones closely resemble

FIG. 2 Clones of cells mutant for *pka* cause ectopic expression of *dpp* and pattern reorganization in the wing. a, Bifurcation of the wing caused by a clone of cells homozygous mutant for *pka*^{po1272} in the anterior compartment. The position of the clone on the dorsal surface is indicated by yellow shading. All *pka* mutant clones that cross the dorsal ventral compartment boundary cause bifurcations ($n=19$). Bifurcations can only be generated by clones that occupy roughly equivalent positions in the dorsal and ventral compartments. In this example, the two surfaces of the supernumerary wing are not in contact in the centre, consequently the veins are not well differentiated except near the base of the wing. b, Detail of the region flanking the clone near the base of the wing in a. The clone occupies a thin strip of tissue, flanked by wild-type cells which form a symmetrical duplication of anterior wing structures. The supernumerary wing veins serve as positional markers (numbered 12321). The endogenous first and second veins are visible at the bottom. c, Detail of the centre of the duplicated wing from a, showing the presence of multiple campaniform sensillae in the clone. Two sensillae are indicated by small arrows. Although this region has not formed a morphologically well-organized vein 3, the presence of a vein in the more proximal part of the wing, and of dorsal campaniform sensillae suggest that cells in this region have adopted vein 3 identity. d, A clone of *pka* mutant cells near the A/P boundary causes little or no phenotype. The position of the clone on the dorsal side is indicated. e, A clone of *pka* mutant cells filling a large portion of both the dorsal and ventral surfaces of the wing in the posterior compartment causes no phenotype. The position of the clone on the dorsal side is indicated. f-i, Clones of *pka* mutant cells in the anterior compartment of the wing cause ectopic expression of a *dpp-lacZ* reporter gene. f, low-magnification view of a wing carrying a large *dpp*-expressing clone. The clone in this example is not marked. i, A marked *pka* clone showing ectopic expression of the *dpp-lacZ* reporter in the anterior compartment of the wing pouch. Cells expressing the *dpp-lacZ* reporter are labelled red (this channel is shown alone in g). The endogenous stripe of *dpp* expression near the compartment boundary is visible as a band of labelled cells. The reporter expresses a cytoplasmic form of β -galactosidase, hence the ring-like appearance of the staining. Cells expressing the Myc epitope tag are shown in green (this channel is shown alone in h). The *pka* mutant cells are identified by the absence of the green label in the nuclei (see inset in i). Note the presence of the adjacent twin-spot clone expressing higher levels of the Myc tag. The *pka* mutant cells express the *dpp-lacZ* reporter in a cell-autonomous manner.

METHODS. Somatic mosaic clonal analysis was carried out using the two lethal P-element alleles described in Fig. 1 legend. The results with the two alleles were identical and are considered together. Clones were induced in larvae heterozygous for *pka* (30C) and a chromosome carry-



ing the dominant Minute mutation M(2L)24F and marked with a f^+ duplication (located at 24F-25A and 30B respectively). Clones of cells in which recombination occurs proximal to 30C will be homozygous for the *pka* mutation and will lack both the recessive cell autonomous trichome and bristle marker f^- and the Minute mutation. Clones were induced by irradiating larvae at 60 ± 12 and 84 ± 12 hours AEL, Minute time (corresponding to second instar; 115 kV, 5 mA for a total dose of 1,000 Rad). To visualize *dpp* expression in mutant clones, a *dpp-lacZ* reporter²⁷ was recombined onto the *pka* mutant chromosome. Clones were marked by the absence of transgene expressing a nuclear P-transposase-Myc epitope tag fusion protein (in a non-Minute background²⁸).

those produced by ectopic expression of *dpp* in the anterior compartment (ref. 9 and F. D.-B. and S.C., unpublished results; K. Basler, personal communication). Clones of *pka* mutant cells located in the endogenous domain of *dpp* expression near the A/P compartment boundary produce little or no perturbation of the wing (Fig. 2d). Clones of *pka* mutant cells in the posterior compartment, where *engrailed* represses *dpp*¹⁸, do not produce any alteration in the pattern of the wing (Fig. 2e).

The similarity in phenotypes of the viable alleles of *pka* and *ptc* suggests that these genes act in a common pathway. Further, clones of cells lacking activity of *pka* or *ptc* lead to ectopic expression of *dpp* in the wing disc¹³ (Fig. 2). But in contrast to the result obtained with *pka* clones, clones of *ptc* mutant cells have not been reported to cause wing bifurcation¹⁶. We re-examined the effects of producing clones of *ptc* mutant cells in the wing disc and noted that the clones of *ptc* mutant cells were on average significantly smaller than the clones of *pka* mutant cells. Bifurcations of the wing were only observed when clones of *pka* mutant cells occupy roughly equivalent positions in the dorsal (D) and ventral (V) compartments. As the dorsal and ventral compartments develop in adjacent territories, it is necessary for a clone to cross the D/V boundary in order to repattern both compartments. Small clones of *pka* mutant cells in either the dorsal or ventral compartment cause local repatterning of the wing in a non-autonomous manner, but do not cause axis bifur-

cation (data not shown). Similar phenotypes are produced by small clones of Hh-expressing cells that do not cross the D/V boundary or by clones of *ptc*^{6P} mutant cells (data not shown).

In the leg imaginal disc the Hh signal directs expression of *dpp* in dorsal-anterior cells and *wg* in ventral-anterior cells². Clones of *hh*-expressing cells in the anterior compartment can cause bifurcation of the leg². Clones of cells mutant for *pka* or for *ptc* in the anterior compartment mimic the effects of ectopic Hh expression and produce axis bifurcation (Fig. 3). The supernumerary legs consist of symmetric duplications of anterior structures (Fig. 3a, b). The clone of mutant cells serves as the axis of symmetry around which the duplications are organized (schematized in Fig. 3f). All mutant clones that cause axis bifurcation run along both the dorsal and ventral surfaces of the supernumerary leg. This property reflects a requirement for the combined activities of *dpp* and *wg* in activating *Distal-less* expression and promoting formation of the proximal-distal axis of the leg (ref. 4 and Fig. 3f). Clones of *pka* or of *ptc* mutant cells that are restricted to the ventral region of the anterior compartment, cause overgrowth rather than axis bifurcation (Fig. 3d, e). Clones of *pka* mutant cells in the posterior compartment of the disc do not produce pattern abnormalities (Fig. 3c).

Here we present evidence that the *Drosophila* homologue of cAMP-dependent PKA is a component of a signal transduction system through which Ptc and Hh control the spatial domain of

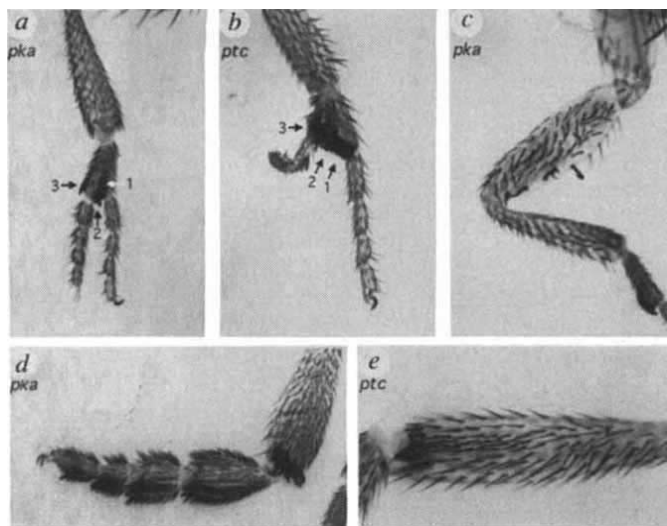
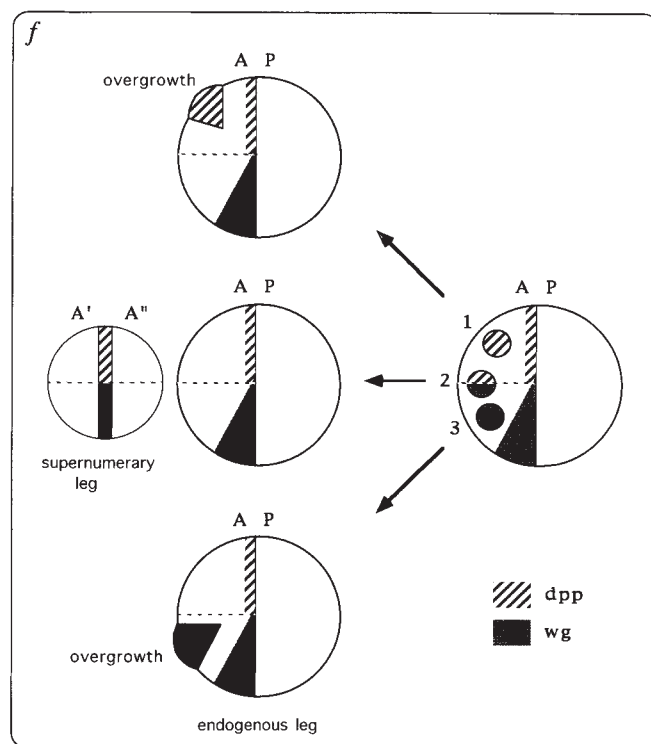


FIG. 3 Clones of cells mutant for *ptc* or *pka* cause pattern reorganization in the leg. a, Bifurcation of a male foreleg caused by a clone of cells mutant for *pka*⁰¹²⁷² in the anterior compartment. The clone occupies both the dorsal and ventral surfaces of the tarsal segments of the supernumerary leg (the mutant phenotype of the bristles is not visible at this magnification). The supernumerary leg consists of a symmetrical duplication of the anterior compartment. Note the presence of a sex comb on each side of the duplicated leg, in addition to the sex comb in the anterior compartment of the endogenous leg (numbered 1–3). b, Bifurcation of a male foreleg caused by a clone of *ptc*^{6P} mutant cells in the anterior compartment. The supernumerary leg consists of a symmetrical, though incomplete, duplication of the anterior compartment, and has two additional sex combs. The clone is both dorsal and ventral. *ptc*^{6P} mutant clones are typically smaller than *pka* mutant clones, and therefore cause less extensive pattern reorganization. c, Clone of *pka* mutant cells in the posterior compartment of the leg producing no phenotype. d, Clone of *pka* mutant cells on the ventral side of the leg. The clone begins in the tibia and extends along the ventral side of the tarsal segments, causing a significant thickening of the leg. The tarsal segments normally have 8 rows of bristles around the circumference. The clone has increased the number of rows of ventral bristles. Although the clone does not cross the dorsal side of the leg, the expansion of the ventral leg caused a non-autonomous expansion



of the dorsal side of the leg as well. Clones of *pka* mutant cells that are exclusively dorsal cause comparable overgrowth, but do not cause axis bifurcation (data not shown), consistent with the requirement for misexpression of both *wg* and *dpp* in order to generate axis bifurcation in the leg⁴. e, Expansion of the ventral tibia produced by a small clone of *ptc*^{6P} mutant cells. The clone produces extra bristle rows, as observed for *pka* mutant clones. The *ptc* mutant clones are typically quite small, and produce more localized effects. f, Scheme representing the relationship between the position of the clones and the effect that the clone has on patterning the leg. Clones that are exclusively on the dorsal side (case 1) or ventral side of the leg (case 3) cause overgrowth, but do not cause bifurcation of the axis. Only clones that occupy both the dorsal and ventral surfaces of the leg disc can generate an ectopic domain of *wg* expression on the ventral side and a corresponding domain of *dpp* expression on the dorsal side of the disc and thereby cause bifurcations (case 2, ref. 4).

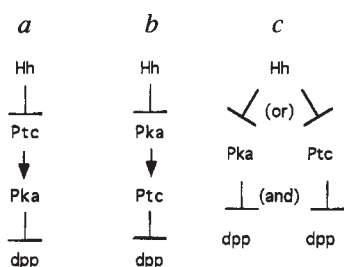


FIG. 4 Alternative models for the relationship between the Hh and Ptc signal transduction pathways. Localized expression of Hh by posterior cells directs expression of *dpp* in adjacent cells near the anterior-posterior compartment boundary of the wing disc^{2,3}. We have presented evidence that the effects of removing PKA are equivalent to the effects of removing Ptc in wing and leg discs. Three models for the relationship between Hh, Ptc and Pka are consistent with these data. *a*, Hh antagonizes the ability of Ptc to activate PKA. *b*, Hh antagonizes the ability of PKA to activate Ptc. *c*, Hh antagonizes either Ptc or Pka, and the activity of both is required to repress *dpp*. Although the location of Ptc in the membrane and PKA in the cytoplasm would seem to favour model *a*, we note that it is not possible to distinguish between these alternatives in the absence of a means to force constitutive activation of one of these genes. For simplicity, only *dpp* is mentioned as a target for repression, but evidence from the embryo and the wing disc suggests that *ptc* and *wg* are also targets for repression^{11,13,14}. We envisage two ways in which the Hh signal might antagonize the ability of Ptc to repress expression of target genes. Hh protein might act directly on the Ptc protein to turn off constitutive signalling^{11,14}. However, examination of the phenotypes of *hh*, *ptc* and *hh ptc* double-mutant embryos has suggested that Ptc does not show all the properties expected of the Hh receptor²⁹. Alternatively, Hh might initiate an independent signal transduction cascade, through an as-yet unidentified Hh receptor (see also ref. 14).

Dpp expression in the wing disc. The properties of *ptc* and *pka* mutant clones are consistent with a role for Ptc and PKA as mediators of the effects of Hh in directing localized expression of *dpp* and *wg* in the leg disc. Removing the activity of *ptc* or *pka* is sufficient to alleviate repression of *ptc* and *dpp* expression in the wing disc (this work and ref. 13). Similarly, removing *ptc* activity alleviates repression of *wg* in the embryo, suggesting that Ptc acts as a constitutively active signalling molecule¹⁴. The Hh signal is thought to direct localized expression of *dpp*, *ptc* and *wg* by antagonizing the ability of Ptc to repress their transcription^{11,13}. The similarity in adult phenotypes and in the effects on gene expression caused by removal of *ptc* or *pka* suggests that these genes act in a common pathway. Given that *ptc* encodes a predicted transmembrane protein^{19,20} and that PKA is a cytoplasmic protein kinase, it is possible that PKA acts downstream to mediate the Ptc-dependent repression (Fig. 4*a*). However, in the absence of more direct genetic tests, we do not exclude the possibility that PKA acts before Ptc in transducing the Hh signal (Fig. 4*b*) or in a parallel pathway (Fig. 4*c*). In other systems cAMP-dependent protein kinases mediate cellular responses to a variety of extracellular signals such as pheromones, growth factors, neuro-transmitters and hormones which serve as ligands for a large family of G-protein-linked receptors (reviewed in refs 21, 22). PKA therefore serves as a component of a signal transduction system that converts information transmitted by intercellular signalling into the changes in gene expression that affect the growth and differentiation of responsive target cells (reviewed in refs 23, 24). PKA has also been implicated in signalling between oocyte and follicle cells in *Drosophila* oogenesis²⁵.

We have shown here that PKA is a component of the signal transduction system through which cell interactions control pattern formation in the developing imaginal discs of *Drosophila*. *Note added in proof*: Similar results have been obtained by the Rubin, Struhl and Kalderon laboratories (*Cell*, in press). □

Received 19 December 1994; accepted 7 February 1995.

1. Diaz-Benjumea, F. J. & Cohen, S. M. *Cell* **75**, 741–752 (1993).
2. Basler, K. & Struhl, G. *Nature* **368**, 208–214 (1994).
3. Tabata, T. & Kornberg, T. *Cell* **76**, 89–102 (1994).
4. Diaz-Benjumea, F. J., Cohen, B. & Cohen, S. M. *Nature* **372**, 175–179 (1994).
5. Irvine, K. & Wieschaus, E. *Cell* **79**, 595–606 (1994).
6. Mohler, J. & Vani, K. *Development* **115**, 957–971 (1992).
7. Lee, J. J., von Kessler, D. P., Parks, S. & Beachy, P. A. *Cell* **71**, 33–50 (1992).
8. Tabata, T., Eaton, S. & Kornberg, T. B. *Genes Dev.* **6**, 2635–2645 (1992).
9. Capdevila, J. & Guerrero, I. *EMBO J.* **13**, 4459–4468 (1994).
10. Ingham, P. W., Taylor, A. M. & Nakano, Y. *Nature* **353**, 184–187 (1991).
11. Ingham, P. W. *Nature* **366**, 560–562 (1993).
12. Schuske, K., Hooper, J. E. & Scott, M. P. *Dev. Biol.* **164**, 300–311 (1994).
13. Capdevila, J., Estrada, M. P., Sánchez-Herrero, E. & Guerrero, I. *EMBO J.* **13**, 71–82 (1994).
14. Forbes, A. J., Nakano, Y., Taylor, A. M. & Ingham, P. W. *Development suppl.* 115–124 (1993).
15. Kalderon, D. & Rubin, G. *Genes Dev.* **2**, 1539–1556 (1988).
16. Phillips, R. G., Roberts, I. J. H., Ingham, P. W. & Whittle, J. R. S. *Development* **110**, 105–114 (1990).
17. Grau, Y. & Simpson, P. *Dev. Biol.* **122**, 186–200 (1987).
18. Rafferty, L. A., Sanicola, M., Blackman, R. K. & Gelbart, W. M. *Development* **113**, 27–33 (1991).
19. Nakano, Y., Guerrero, I., Hidalgo, A., Whittle, J. R. S. & Ingham, P. W. *Nature* **341**, 508–513 (1989).
20. Hooper, J. & Scott, M. P. *Cell* **59**, 751–765 (1989).
21. Watson, S. & Arkininstall, S. *The G-Protein Linked Receptors* (Academic, London, 1994).
22. Stradler, C. D., Fong, T. M., Tota, M. & Underwood, D. A. *Rev. Biochem.* **63**, 101–132 (1994).
23. Devreotes, P. *Neuron* **12**, 235–241 (1994).
24. Hartwell, L. *Nature* **371**, 286 (1994).
25. Lane, M. E. & Kalderon, D. *Genes Dev.* **8**, 2986–2995 (1994).
26. Török, T., Tick, G., Alvarado, M. & Kiss, I. *Genetics* **135**, 71–80 (1993).
27. Blackman, R. K., Sanicola, M., Rafferty, L. A., Gillevet, T. & Gelbart, W. M. *Development* **111**, 657–665 (1991).
28. Xu, T. & Rubin, G. M. *Development* **117**, 1223–1237 (1993).
29. Bejsovec, A. & Wieschaus, E. *Development* **119**, 501–517 (1993).

ACKNOWLEDGEMENTS. We thank E. McDonald for calling to our attention the mutant phenotype of the P113/2 strain; D. Strutt, V. Wiersdorff and M. Mlodsk for sharing data and the P89/18 mutant before publication; I. Kiss, T. Lavery and K. Matthews for P-element stocks; I. Guerrero, P. Beachy, T. Tabata, T. Kornberg, L. Maves and L. Johnston for reagents; and the Soriano, Foote, Laird, Hockenbery and Tapscott laboratories for support during screening of the P-element collection. We thank our colleagues at the FHCRC and EMBL for sharing equipment and reagents, for discussion and comments on the manuscript. This work was supported by the EMBL (S.C. and F.D.-B.); the CNRS (T.L.); the NIH, an ACS Junior Faculty Research Award, and a Basil O'Connor Starter Scholar Research Award (S.M.P.). S.M.P. is a Pew Scholar in the Biomedical Sciences.

Molecular characterization of eukaryotic polysialyltransferase-1

Matthias Eckhardt, Martina Mühlenhoff, Andrea Bethe, Jaap Koopman*, Matthias Frosch & Rita Gerardy-Schahn†

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, 30625 Hannover, Germany

* Department of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

POLYSIALIC acid (PSA) is a dynamically regulated product of post-translational modification of the neural cell adhesion molecule, NCAM^{1,2}. Presence of the large anionic carbohydrate modulates NCAM binding properties and, by increasing the intercellular space, influences interactions between other cell surface molecules^{1–5}. PSA expression underlies cell type- and developmental-specific alterations⁶ and correlates with stages of cellular motility^{6–8}. In the adult, PSA becomes restricted to regions of permanent neural plasticity and regenerating neural and muscle tissues^{6,9,10}. Recent data implicate its important function in spatial learning and memory^{11,12}, and in tumour biology^{13–16}. Here we describe the molecular characterization of polysialyltransferase-1, the key enzyme of eukaryotic PSA synthesis. In reconstitution experiments, the newly cloned enzyme induces PSA synthesis in all NCAM-expressing cell lines. Our data therefore represent convincing evidence that the polycondensation of α -2,8-linked sialic

† To whom correspondence should be addressed.

acids in mammals is the result of a single enzymatic activity and provide a new basis for studying the functional role of PSA in neuro- and tumour biology.

Studies aimed at enlightening the biosynthetic pathway and the regulation of PSA synthesis suggest the concerted activity of several specific enzymes located within the Golgi apparatus^{17,18}. Therefore, as a first step, it was important to determine how many enzymatic activities are involved in conferring PSA to its acceptor molecule NCAM. Chinese hamster ovary (CHO) cells, which are positive for NCAM and PSA (ref. 19 and Fig. 1a), were used for a complementation analysis. PSA-loss mutants, isolated by negative selection on the PSA-specific monoclonal antibody 735 (ref. 20), were analysed for intact NCAM surface expression (Fig. 1a), shown to express α -2,3-linked sialic acid in the immunoprecipitated NCAM molecule (Fig. 1b), and then were used for fusion experiments. Surprisingly, only one single complementation class (polysialyltransferase-Cl; PST-Cl) could be identified with 40 individual clones selected from 9 independent mutagenesis experiments. In order to rule out the possibility that the mutation introduced in these clones is of a dominant phenotype, control fusions were done between the PST-Cl clone CHO-2A10 and either CHO-wild-type (CHO-wt) cells or the clone CHO-1E3, which is defective in sialic acid transport and therefore does not express sialylated proteins (M.E. *et al.*, manuscript in preparation). In hybrid cells from both fusions, PSA surface expression was detectable (Fig. 1a). These data strongly suggest that the polycondensation of α -2,8-linked sialic acids in mammals is mediated by a single enzyme, which is mutated in clones of the complementation class PST-Cl.

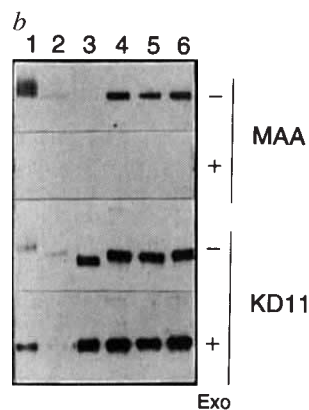
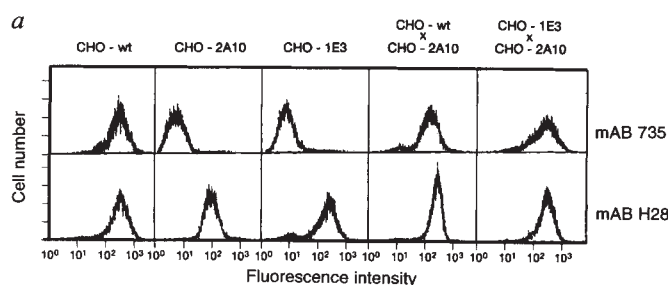
To isolate the gene defective in PST-Cl clones, the mutant clone CHO-2A10 was used for expression cloning. A complementary DNA library from CHO-wt cells (in pCDM8) together with the vector pPSVE1-PyE carrying the polyoma large T-antigen²¹ was transfected by electroporation. PSA-positive cells were collected by panning²² on antibody 735 (refs 20, 23), and plasmid DNA was subsequently extracted and amplified in *Esch-*

erichia coli MC1061/p3 (ref. 22). After three cycles of transfection and panning, the cDNA encoding PST-1 was enriched to about 1:10³ and could be finally isolated by sibling selection. We termed the cDNA clone pEPST-ME7 (for eukaryotic polysialyltransferase clone ME7). It contained an insert of 2,026 base pairs (bp) and an open reading frame of 1,080 bp potentially encoding a protein of 359 amino acids with a predicted M_r of 41.2 K. The primary sequence (Fig. 2a) shows the characteristic features of the sialyltransferase family, including the two sialyl motifs²⁴. The requirements of a type II transmembrane protein²⁵ are only partially fulfilled. A stretch of 13 hydrophobic amino acids (at least 17 are required) was found within the N-terminal domain of the molecule. Nevertheless, it is likely that this truncated hydrophobic motif represents a Golgi-retention signal, because the cationic borders characteristic for type II transmembrane proteins were also found. Comparison of the PST-1 primary sequence with the primary sequences of other sialyltransferases²⁶⁻²⁸ revealed that the similarity is predominantly concentrated to the sialyl motifs L and S²⁴. Analysis as in ref. 29 produced the dendrogram shown in Fig. 2b. The highest degree of homology is found with STX (59%), a sialyltransferase of unknown specificity²⁶ and with the recently cloned GD₃ synthase (28%), the only α -2,8-sialyltransferase identified so far^{27,28}. Partial sequences obtained by polymerase chain reaction (PCR) from the human and mouse PST-1 genes demonstrated an extensive sequence homology to the hamster gene (data not shown).

Transient expression of pEPST-ME7 cDNA in different cell lines of the phenotype NCAM⁺/PSA⁻ (mutant clone CHO-2A10, NIH-3T3 and COS-hN-6), resulted in surface expression of PSA (Fig. 3a). As shown in Fig. 3b, PSA in these transfectants was attached to NCAM immunoprecipitated by an anti-NCAM serum. Pretreatment of the cells with endoneuraminidase NE (endo NE; ref. 30) in both assay systems completely abolished the reactivity with antibody 735. After endo NE treatment, the

FIG. 1 a, FACS-analysis of CHO-wt and mutant clones. Staining with the mouse-NCAM specific mAb H28 indicates that NCAM surface expression in the mutant cell lines CHO-2A10 (PST-Cl) and CHO-1E3 (defective in sialic acid transport) is almost identical to that observed in CHO-wt cells. In contrast, the reactivity with the PSA-specific mAb 735 (refs 20, 23) is restricted to the CHO-wt cells. To obtain the hybrid cells CHO-2A10 × WT and CHO-2A10 × CHO-1E3, we introduced a neomycin resistance gene into clone CHO-2A10 and a hygromycin resistance gene into CHO-wt and CHO-1E3 cells, respectively. Equal amounts of the two relevant cell types were plated into cell culture dishes and cell fusion was induced by polyethyleneglycol (50% PEG 1500 in 74 mM HEPES buffer, pH 8.0). Double-positive hybrids were selected with G418 and hygromycin. Although the NCAM signal in hybrid cells was identical to that observed in parental cells, both fusion products expressed the 735 epitope. b, To ensure that the mutants used for the complementation analysis express α -2,3-linked sialic acid, NCAM samples (immunoprecipitated with a polyvalent antiserum) from CHO-wt cells and chemically induced mutants were analysed with the *Maackia amurensis* lectin (MAA, Boehringer Mannheim), which specifically recognizes sialic acid in α -2,3-linkage to galactose. NCAM samples are shown before (–) and after (+) treatment with exoneuraminidase (*Clostridium perfringens*, Boehringer Mannheim). Because of polysialylation, both MAA and the anti-NCAM mAb KD11 (ref. 31) recognized microheterogenous bands in CHO-wt cells (lanes 1 and 2; wt-cells in two concentrations), whereas discrete protein bands became visible in PST-Cl mutants (lanes 4–6; 3 individual mutants: 2A10, 1H8 and 3A7). In contrast, mutant CHO-1E3, which is defective in sialic acid transport did not react with MAA (lane 3) and, in accordance with the deficiency of sialylated proteins, the polypeptide band recognized by KD11 in this mutant, showed a significantly reduced M_r . Treatment with exoneuraminidase abolished MAA reactivity completely and converted the protein bands recognized by mAb KD11 into forms with identical M_r .

METHODS. Cells (1×10^7) were collected, membrane proteins extracted with lysis buffer (20 mM Tris-HCl, pH 8; 1% nonidet P 40) and NCAM was immunopurified on a Protein A-bound anti-NCAM antibody (mAb



KD11). Samples were separated by 7.5% SDS-PAGE, blotted onto nitrocellulose membranes, and developed in parallel with either biotinylated MAA or anti-NCAM mAb KD11. Protein bands were visualized using alkaline phosphatase conjugated streptavidin.

a

```

1  GCGACCGCCACCTCCAATGCACAAGGTGTACATTTGAAAAGAAACCTGAGCCGGGGGA
61  GAAGCGCTGAGAGACCTGGCTGGCTAGTGCACAACTGCGCGGCGAGGCGCTGGGCAGC
121 CTGGAGAACCCAGAGAGCTCCACGCGAGACCATCTAGCGACCTGATTCTGGGATCTCGG
181 CTCACGCTCCCTTCGCAATTTTCAGATTCTCTCCCTCGATTATTTCCCAAAACGGAA
241 CCTTTATACCAAGAGAGGTGCGGAGCTGGGCGAACCGAGACTTTCCCGGCGACCAAG

301 ATGCGCTCCATTAGAAAACGGTGGACCATCTGCACTATAAGTCTACTTCTGATCTTTTAT
1  MetArgSerIleArgLysArgTrpThrIleCysThrIleSerLeuLeuLeuIlePheTyr

361 AAGACAAAAGAGATAGCCAGAAGTGGAGACCAAGAGACGCAATCATCGGAGATGGT
21  LysThrLysGluIleAlaArgThrGluGluHisGlnGluThrGlnLeuIleGlyAspGly

421 GAATTGTGTTTGGAGAGATCACTTGTCAACAGCTCTGATAAAATCATTGGAAGGCTGGC
41  GluLeuCysLeuSerArgSerLeuValAsnSerSerAspLysIleIleArgLysAlaGly

481 TCAACCATCTTCCAACATTCTGTACAAGGCTGGAGAAATCAATCTCTTCTTAGTCTGGAG
61  SerThrIlePheGlnHisSerValGlnGlyTrpArgIleAsnSerSerLeuValLeuGlu

541 ATACGGAAGAATCTCCGTTTCTTAGATGCTGAACGTGATGTCTCTGTGGTCAAGAGC
81  IleArgLysAsnIleLeuArgPheLeuAspAlaGluArgAspValSerValValLysSer

601 AGCTTCAAGCCTGGTGTATGTCATCCACTATGTGTGGACAGACGCGGAGCGTAAATATT
101 SerPheLysProGlyAspValIleHisTyrValLeuAspArgArgThrLeuAsnIle

661 TCCCATGATCTGCACAGCCTCTGCCTGAAGTTTACCAATGAAAACCGCAGGTTTAAAG
121 SerHisAspLeuHisSerLeuLeuProGluValSerProMetLysAsnArgArgPheLys

721 ACCTGTGCTGTTTGGAAACTCTGGCATTCTACTAGACAGTGGATGTGGCAAGGAGATT
141 ThrCysAlaValValGlyAsnSerGlyIleLeuLeuAspSerGlyCysGlyLysGluIle

781 GACAGTCACAATTTTGTAACTAGGTGCAATCTAGCTCTGTGGTGGAGTTTGTGCGGAT
161 AspSerHisAsnPheValIleArgCysAsnLeuAlaProValValGluPheAlaAlaAsp

841 GTGGGACTAAATCAGATTTTATTACCATGAACCCATCAGTTGTGCAGAGAGCATTTTGA
181 ValGlyThrLysSerAspPheIleThrMetAsnProSerValValGlnArgAlaPheGly

901 GGCTTTCGGAATGAGAGTGACAGAGCAAAATTTGTGCATAGACTTCCATGCTGAATGAC
201 GlyPheArgAsnGluSerAspArgAlaLysPheValHisArgLeuSerMetLeuAsnAsp

961 AGTGTCTTGGATCCCCGCTTTCATGGTCAAAGGAGGAGAGAAGCAGTGAATGGGTT
221 SerValLeuTrpIleProAlaPheMetValLysGlyGlyGluLysHisValGluTrpVal

1021 AATGCATTAACTCTTAAAGAACAGCTGAAAGTGCGAACTGCCTATCCATCACTGAGACTT
121 AsnAlaLeuIleLeuLysAsnLysLeuLysValArgThrAlaTyrProSerLeuArgLeu

1081 ATTATGCTGTCTAGAGGTTACTGGCTGACCAACAAAGTGCCCATCAAAGACCCGACACA
261 IleHisAlaValArgGlyTyrTrpLeuThrAsnLysValProIleLysArgProSerThr

1141 GGCTCTCATGTACACACTGGCCACAGATTTTGTGATGAAATTCACCTGTATGGGTTTC
281 GlyLeuLeuMetTyrThrLeuAlaThrArgPheCysAspGluIleHisLeuTyrGlyPhe

1201 TGGCCCTTCCCTAAGGATTTGAATGGAAAAGCTGTGAAATATCATTACTACGATGACTTG
301 TrpProPheProLysAspLeuAsnGlyLysAlaValLysTyrHisTyrTyrAspAspLeu

1261 AAATATAGATACTTTTCCAACGCAAGCCCTCACAGAATGCCATTAGAATTCAAAACCCCTG
321 LysTyrArgTyrPheSerAsnAlaSerProHisArgMetProLeuGluPheLysThrLeu

1321 AATGTGTACACACAGAGGAGCACTAAAACAGCCACAGGGAAGTGCATGAAGCAATAA
341 AsnValLeuHisAsnArgGlyAlaLeuLysLeuThrThrGlyLysCysMetLysGln

1381 AGCACAATTTGAAGGATCAAACTGGATAGAACTTTTCTAAAGATGCTTCTGGAGATT
1441 TAGAAAACAGGATCAAAAACAGGCTGGGTTTTCAGCATCCACTGACTGAATAGCTGAAA
1501 TGAAGTCCATGGGAATCCACCACAGCTGATGAAATACCTGCGCAAGTGTCTTAACATA
1561 AAATATCTGACTTCAAGGTCCTAGTAAGTGCACCTCCACGAAAGATACAGTTTGAAT
1621 GTATTATCAGTAGTGTTTACAAGATCCAAGTGCACCTCATCATTAAATAGCAAAGCAA
1681 TATGTTCTGCTACTGTGGGCGACCGCTGTAATGCCAAGCAGCACTGGAAGAGGAACTCAGG
1741 AGCATCAGACTCGGAGCTTGGGAAATTAACATCTTATCCGAGAAATGAAGAAGAAAA
1801 AGAATTCAACAGTGAATCCATGAGATGAAGTAACCTGAAGGAATGTCTTCAGTCAGGA
1861 CACTGAGAGTGATCATGTGTGTGTTTGTCTGTGTTTGTGTTCTTCTGAACTTGTT
1921 TTCTTTGGGTATGGGTGAATAGAAATTCATCTGAGGTACAGAAATGGGAAATACATGA
1981 CAGAGAAAATAAACATCAAAACAGTCAAAAAAAGAAAAAAGAAAAA

```

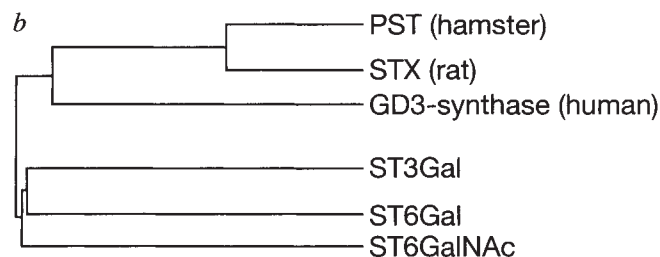


FIG. 2 **a**, Nucleotide and deduced protein sequence of the pEPST-ME7 cDNA. The clone of 2 kb isolated from CHO-wt cells was sequenced on both strands by the dideoxy chain termination method using T7 DNA polymerase sequencing kit (Pharmacia) with the aid of specific oligonucleotide primers. The hydrophobic stretch of 13 amino acids, and the sialyl motifs L (amino acids 141–183) and S (amino acids 278–300) are underlined. Potential *N*-glycosylation sites are indicated by asterisks. This sequence will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Data Libraries under the accession number Z46801. **b**, The dendrogram²⁹ describes the relationship of PST-1 to other cloned sialyltransferases. The branching pattern indicates that PST-1 forms one group together with STX (ref. 26) and a second α -2,8-sialyltransferase the G_{D3} -synthase^{27,28}. The data suggest that also STX may be a sialyltransferase that transfers sialyl residues in α -2,8-linkage to carbohydrate acceptors.

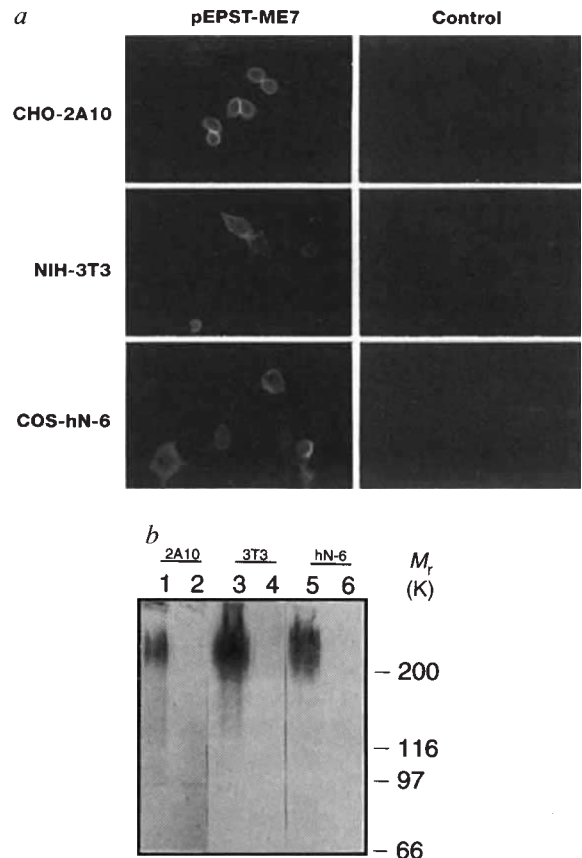


FIG. 3 **a**, Transient expression of pEPST-ME7 cDNA in the mutant clone CHO-2A10, in NIH-3T3 cells (ATCC, CRL 1658), and in COS-hn-6 (COS-M6 clone stably transfected with human NCAM-140 cDNA³¹ in pRc/CMV (Invitrogen); the NCAM construct is devoid of alternatively spliced exons VASE and MSD) resulted in surface expression of PSA as shown by immunofluorescence using mAb 735. **b**, The PSA detectable on the surface of pEPST-ME7 transfectants is bound to NCAM. Cells with the phenotype NCAM⁺/PSA⁺ were transfected with pEPST-ME7 or pCDM8 alone as described above, collected and solubilized in lysis buffer (see Fig. 1). NCAM was immunoprecipitated from the lysates using an anti-NCAM serum, and the samples were analysed by western blot (as described in Fig. 1) with mAb 735. Microheterogenous bands became visible in PST transfectants (lane 1, CHO-2A10; lane 3, NIH-3T3; lane 5, COS-hn-6) indicating that NCAM in these cells is polysialylated. In contrast, control transfection with pCDM8 did not result in the appearance of mAb 735 epitopes (lane 2, CHO-2A10; lane 4, NIH-3T3; lane 6, COS-hn-6).

METHODS. **a**, Tissue culture dishes were seeded with freshly trypsinized cells 18 h before transfection with 2 μ g pEPST-ME7 or an equal amount of the empty vector pCDM8, using lipofectamine (Gibco BRL). Transfected cells were incubated 62 h later with the PSA specific mAb 735 (ref. 20) for 30 min at room temperature, and specifically bound mAb was detected with fluorescein-conjugated anti-mouse Fab fragments (Dianova).

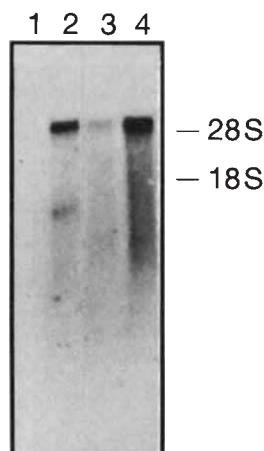


FIG. 4 The northern blot analysis of pEPST-ME7 transcripts revealed a single band of 5.2 kb in the PSA-positive CHO-wt cells (lane 2), and in adult (lane 3) and postnatal day 1 mouse brain (lane 4). In accordance with the reduced PSA expression in adult brain, a very faint band was obtained (lane 3). No signal was found with PSA-negative NIH-3T3 cells (lane 1).

METHODS. Poly(A)⁺ RNA samples (3 µg each lane) were blotted onto nylon membranes and analysed with a digoxigenin (Boehringer Mannheim)-labelled RNA-probe that contained the entire coding region of pEPST-ME7. Final washing conditions were 0.1 × SSC, 0.1% SDS and 65 °C.

staining patterns in Fig. 3a, b were identical to those observed in control transfections with pCDM8 (data not shown).

In northern blot analysis, a PST-1-specific cDNA probe recognized a single transcript of 5.2 kb in CHO-wt cells, in adult and in embryonic mouse brain (Fig. 4, lanes 2–4). However, according to the restricted expression of PSA, the signal is drastically reduced in adult mouse brain (lane 3). NIH-3T3 cells, which are negative for PSA, gave no hybridization signal (lane 1). These results imply that the PST-1 activity is regulated on the messenger RNA level and, furthermore, suggest PST-1 to be the cellular control element regulating PSA synthesis. Southern blot analysis of hamster and mouse genomic DNA using the PST-1 cDNA as a probe, revealed a restriction pattern for the PST-1 gene, consistent with the presence of a single-copy gene (data not shown).

Although, the final determination of the number of enzymes involved in eukaryotic PSA synthesis is still awaited, our data strongly suggest that PST-1 is the only factor unique in PSA-positive cells. Moreover, because the polycondensation of sialic acid is the final step in the maturation of polysialylated NCAM, panning (see ref. 22) on antibody 735 should provide a useful method to identify all enzymes being prerequisite in the synthesis and surface expression of PSA, if appropriate mutants become available. □

Received 22 November 1994; accepted 24 January 1995.

- Hoffman, S. & Edelman, G. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5762–5766 (1983).
- Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. L. & Sunshine, J. *Science* **240**, 53–57 (1988).
- Acheson, A., Sunshine, J. L. & Rutishauser, U. *J. Cell Biol.* **114**, 143–153 (1991).
- Yang, P., Yin, X. & Rutishauser, U. *J. Cell Biol.* **116**, 1487–1496 (1992).
- Doherty, P., Cohen, J. & Walsh, F. S. *Neuron* **5**, 209–219 (1990).
- Edelman, G. M. *A. Rev. Cell Biol.* **2**, 81–116 (1986).
- Tang, J., Landmesser, L. & Rutishauser, U. *Neuron* **8**, 1031–1044 (1992).
- Landmesser, L. *J. Neurobiol.* **23**, 1131–1139 (1992).
- Martini, R. & Schachner, M. *J. Cell Biol.* **106**, 1735–1746 (1988).
- Daniiloff, J. K., Levi, G., Grumet, M., Rieger, F. & Edelman, G. M. *J. Cell Biol.* **103**, 929–945 (1986).
- Cremer, H. et al. *Nature* **367**, 455–459 (1994).
- Tomasiewicz, H. et al. *Neuron* **11**, 1163–1174 (1993).
- Moolenaar, C. E. C. et al. *Cancer Res.* **50**, 1102–1106 (1990).
- Takamatzu, K. et al. *Cancer Res.* **54**, 2598–2603 (1994).
- Komminoth, P., Roth, J., Lackie, P. M., Bitter-Suermann, D. & Heitz, P. U. *Am. J. Pathol.* **139**, 297–304 (1991).

- Kern, W. F., Spier, C. M., Miller, T. P. & Grogan, T. M. *Leukemia Lymphoma* **12**, 1–10 (1993).
- McCoy, R. D., Vimr, E. R. & Troy, F. A. *J. Biol. Chem.* **260**, 12659–12699 (1984).
- Kitazume, S., Kitajima, K., Inoue, S., Inoue, Y. & Troy, F. A. *J. Biol. Chem.* **269**, 10330–10340 (1994).
- Block, R. J. *J. Cell Biol.* **116**, 449–463 (1992).
- Frosch, M., G6rgen, I., Boulnois, G. J., Timmis, K. N. & Bitter-Suermann, D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1194–1198 (1985).
- Bierhuizen, M. F. A., Mattei, M.-G. & Fukuda, M. *Genes Dev.* **7**, 468–478 (1993).
- Seed, B. & Aruffo, A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3365–3369 (1987).
- Häyrynen, J., Bitter-Suermann, D. & Finne, J. *Molec. Immun.* **26**, 523–529 (1989).
- Drickamer, K. *Glycobiology* **3**, 2–3 (1993).
- Klein, P., Kanehisa, M. & DeLisi, C. *Biochim. biophys. Acta* **815**, 468–471 (1985).
- Livingston, B. D. & Paulson, J. C. *J. Biol. Chem.* **268**, 11504–11507 (1993).
- Nara, K. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 7952–7956 (1994).
- Sasaki, K. et al. *J. Biol. Chem.* **269**, 15950–15956 (1994).
- Higgins, D. G. & Scharp, P. M. *Gene* **73**, 237–244 (1988).
- Tomlinson, S. & Taylor, P. W. *J. Virol.* **55**, 374–378 (1985).
- Gerardy-Schahn, R. & Eckhardt, M. *Int. J. Cancer* **8** (suppl.), 38–42 (1994).

ACKNOWLEDGEMENTS. We thank D. Bitter-Suermann for continuous support, B. Lüscher, U. Vogel and M. Cahill for critical remarks on the manuscript, E. Bock and C. Goridis for generously providing antibodies, J. Westermann for providing the FACScan and for many helpful discussions and M. Fukuda for kindly providing the vector pPSVE1-PyE. This work was supported by grants from the Deutsche Forschungsgemeinschaft. J.K. was supported by grants from the Dutch Cancer Society. M.F. is supported by a professorship from the Hermann- and Lilly-Schilling Foundation.

Failure of a single-headed kinesin to track parallel to microtubule protofilaments

Elise Berliner*, Edgar C. Young†, Karin Anderson†, Hansraj K. Mahtani† & Jeff Gelles†§

* Biophysics Program, † Graduate Department of Biochemistry and ‡ The Center for Complex Systems, Brandeis University, 415 South Street, Waltham, Massachusetts 02254, USA

KINESIN, a two-headed motor enzyme molecule, hydrolyses ATP to direct organelle transport along microtubules. As it moves along a microtubule, kinesin remains associated with, or ‘tracks’, microtubule protofilaments^{1,2}. We have prepared truncated kinesin derivatives that contain either two mechanochemical head domains³ or only a single head. Unlike intact kinesin and the two-headed derivatives, the one-headed enzyme frequently fails to track protofilaments, suggesting that it detaches from microtubules during movement. In this way, the one-headed kinesin derivative is similar to the motor enzyme myosin, which frequently detaches from the actin filament during movement⁴. For myosin (which has two heads), the consequence of this detachment is that single molecules do not appear to drive continuous movement along the filament⁵. Our observations suggest that the ability of single two-headed kinesin molecules to drive continuous movement^{6,7} results from a ‘hand-over-hand’ mechanism^{6–8} in which one head remains bound to the microtubule while the other detaches and moves forwards.

We prepared kinesin derivatives K448-BIO, K401-BIO and K340-BIO, which respectively contain 448, 401 and 340 N-terminal residues of the *Drosophila* kinesin α -chain linked to 87 residues of the *Escherichia coli* biotin carboxyl carrier protein (BCCP). The BCCP domain is post-translationally biotinylated *in vivo*^{9,10}, facilitating study of the derivatives in motility assays in which the enzyme molecules are specifically attached to streptavidin-coated polystyrene beads. Gel filtration and sedimentation velocity studies (Table 1) demonstrate that K448-BIO and K401-BIO form dimers in solution, whereas K340-BIO exists as a monomer.

All three derivatives move streptavidin-coated microscopic polystyrene beads along microtubules (Fig. 1). Mean velocities of K448-BIO and K401-BIO are $760 \pm 70 \text{ nm s}^{-1}$ and

§ To whom correspondence should be addressed.

$610 \pm 160 \text{ nm s}^{-1}$ respectively, which are similar to the velocity of intact kinesin. K340-BIO moves at $170 \pm 50 \text{ nm s}^{-1}$, similar to the highest velocity measured for kinesin derivatives containing only the 339 N-terminal residues¹¹. Formation of a two-headed (dimeric) solution structure is thus unnecessary for kinesin movement. However, we cannot exclude the possibility that multiple one-headed molecules that lie closely apposed on the bead surface interact to produce movement.

To examine the pattern of enzyme movements over the microtubule surface lattice, we followed two-dimensional projections of the trajectories of enzyme-bead conjugates using a nanometre-precision bead-tracking procedure¹ (Fig. 1). Beads linked to the two-headed K448-BIO or K401-BIO deviate from the microtubule axis less than $\sim 20 \text{ nm}$ in 200 nm of axial motion (Fig. 1a). Because protofilaments are oriented nearly parallel to the microtubule axis¹², this implies that these derivatives track a protofilament or a pair of protofilaments as they move¹³, as does the intact molecule^{1,2}. Rarely observed abrupt deviations of $\sim 50 \text{ nm}$ (Fig. 1a, arrows) are consistent with the movement of $\leq 37 \text{ nm}$ expected when a kinesin molecule moves to an adjacent protofilament (Fig. 1a, inset).

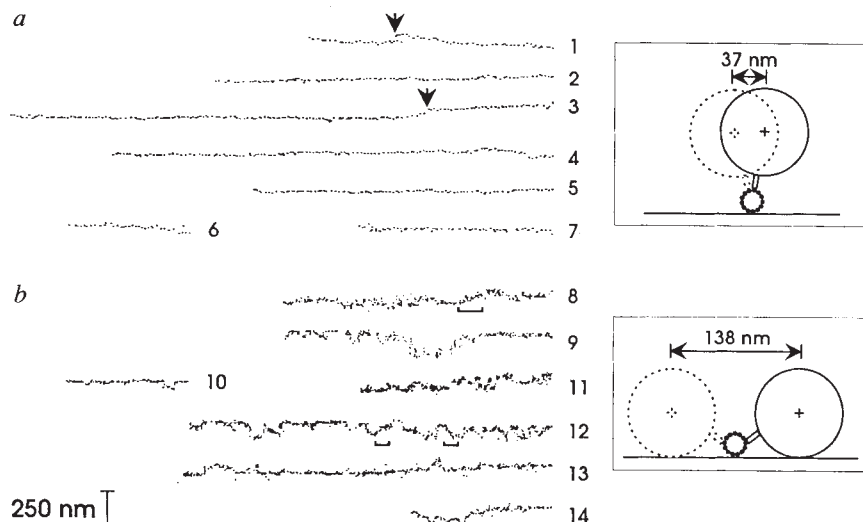
In contrast to the behaviour of the two-headed enzyme, beads moved by the one-headed K340-BIO (Fig. 1b) exhibit frequent large deviations from the microtubule axis. Perpendicular movements are typically up to $\sim 160 \text{ nm}$, consistent with the expected movement of 138 nm in a model where the enzyme is capable of movement over the entire accessible microtubule surface (Fig. 1b, inset), and they have a 'wandering' character reminiscent of a random walk constrained between barriers. (Beads driven by cytoplasmic dynein exhibit similar behaviour¹⁴.) Indeed, the minority of K340-BIO records that display movements largely

parallel to the microtubule axis have perpendicular deviations largely on one side of the trace (Fig. 1b, traces 9 and 13), possibly because movement in the opposite direction is blocked by collision of the bead with the coverslip. Quantitative analysis confirms that mean perpendicular motion of beads driven by K340-BIO is significantly greater than that of beads driven by the two-headed derivatives (Fig. 2; $P < 0.0005$ (*t*-test, $n = 58$)). The perpendicular movement of K340-BIO beads suggests that the enzyme molecules do not maintain their association with a particular protofilament (or protofilament pair) during movement.

It might be argued that the perpendicular motion of the K340-BIO beads is simply brownian motion of beads linked to the microtubule by a structure more flexible than that obtained with two-headed enzymes. However, the initial slopes of mean-square perpendicular displacement against time curves¹⁵ (not shown) are $\sim 10^3$ -fold smaller than the Stokes' law diffusion coefficient of the bead. Therefore, the slow perpendicular movements cannot be explained by brownian motion of the bead linked to a fixed circumferential position on the microtubule by a flexible or elastic tether. Also, most K340-BIO beads bound to microtubules in the absence of ATP display only limited ($\pm \sim 6 \text{ nm}$ r.m.s.) perpendicular motion.

Protofilament tracking was not impeded in experiments in which the K448-BIO concentration was reduced to one enzyme molecule per bead (Fig. 1a, traces 6 and 7). Similarly, protofilament tracking by intact kinesin is not affected by changes in the number of molecules driving movement². Therefore, the failure of K340-BIO beads to track cannot be attributed simply to a reduction in the number of molecules driving the observed movements. In experiments with nominally one K340-

FIG. 1 Movement along microtubules of $0.10 \mu\text{m}$ diameter beads conjugated to two-headed and one-headed kinesin derivatives at 1.6 – 2.6 mM ATP. Position of each of 14 beads (traces 1–14) is plotted at 33-ms intervals. Traces are oriented so that the microtubule is horizontal. a, Two-headed kinesin derivatives K401-BIO (traces 1–3) and K448-BIO (traces 4–7). For traces 6 and 7, the K448-BIO concentration was ~ 1 molecule per bead; higher concentrations were present for traces 4 and 5. Arrows indicate rare events in which beads make abrupt $\sim 50\text{-nm}$ movements perpendicular to the microtubule axis. Inset: cross-sectional diagram showing the largest expected displacement (37 nm) of the centre of a 100-nm bead (large circles) when a kinesin-streptavidin crossbridge (rectangle) moves its point of attachment to an adjacent microtubule protofilament (small circles), assuming a 14-nm crossbridge length (10 nm for the kinesin head²³ plus 4 nm (determined from a structure²⁴ in the Brookhaven Protein Data Bank) for streptavidin). b, One-headed kinesin derivative K340-BIO. Brackets, examples of segments in which beads move continuously for $>500 \text{ ms}$ in a straight path oriented at a large angle to the microtubule axis. Inset: Diagram showing the expected displacement when circumferential crossbridge movement is unrestricted. In this case, the range of movement (138 nm) is limited only by bead collision with the coverslip surface (horizontal line).



METHODS. Streptavidin-coated beads were prepared by first conjugating 0.01 mg ml^{-1} biotin-x-cadaverine (Molecular Probes) to 0.25% (w/v) 100-nm carboxylated polystyrene beads (Seradyn) with 0.5 mg ml^{-1} 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, 0.625 mg ml^{-1} N-hydroxysulphosuccinimide²⁵ in 10 mM sodium phosphate, $\text{pH } 7.0$, 0.1% Tween-20. After 1.5 h , the reaction was quenched by addition of 0.2 M glycine, $\text{pH } 8.0$. Excess biotin-x-cadaverine was removed by dialysis in 10 mM sodium phosphate, $\text{pH } 7.0$, 50 mM NaCl, 0.1% Tween-20. Streptavidin was then added to 20 mg g^{-1} of beads and the beads were stored at 4°C . Before use, unbound streptavidin was removed by gel filtration (Sephacrose CL-4B) in 50 mM imidazole-Cl, $\text{pH } 6.7$, 50 mM

NaCl, 2 mM EGTA, 4 mM MgCl_2 and 0.1% Tween-20. Final bead concentration was determined by comparing A_{260} to that of standard dilutions of unconjugated beads. For motility assays, microtubules were prepared as described¹⁰. Streptavidin-coated beads were incubated with 1 mg ml^{-1} BSA (bovine serum albumin), 1 mM dithiothreitol (DTT) for 30 min before the addition of the kinesin derivatives. All reagents for motility assays were diluted in ABT (50 mM imidazole-Cl, $\text{pH } 6.7$, 50 mM KCl, 2 mM EGTA, 4 mM MgCl_2 , $20 \mu\text{M}$ taxol). Typically, flow cells¹⁰ were rinsed with 0.01 – 0.08 mg ml^{-1} microtubules, incubated for 1 min , rinsed three times with ABT, rinsed with 0.8 mg ml^{-1} α -casein, incubated for 5 min , rinsed with 1 mg ml^{-1} BSA, incubated 5 min and rinsed twice with 1 mg ml^{-1} BSA, 1.6 – 2.6 mM ATP; rinse volumes were $10 \mu\text{l}$. Beads conjugated with the kinesin derivatives were then diluted in 1 mg ml^{-1} BSA, 1.6 – 2.6 mM ATP and added to the flow cell. Flow cells were examined at 25°C by video differential interference contrast microscopy²⁶. Images were recorded and processed with hardware²⁷ and algorithms¹ used previously.

TABLE 1 Oligomeric state of biotinylated kinesin derivatives

Derivative	$10^7 \times D_{20,w}$ (cm ² s ⁻¹)*	$s_{20,w}$ (S)†	$\bar{v}$ (ml g ⁻¹)‡	M_r §	Subunit M_r	Oligomeric state
K448-BIO	3.7 ± 0.2	4.8 ± 0.1	0.737	12.1 ± 0.8 × 10 ⁴	5.9 × 10 ⁴	Dimer
K401-BIO	4.7 ± 0.3	4.8 ± 0.1	0.738	9.7 ± 1.4 × 10 ⁴	5.5 × 10 ⁴	Dimer
K340-BIO	6.9 ± 0.5	3.3 ± 0.1	0.739	4.4 ± 0.5 × 10 ⁴	4.7 × 10 ⁴	Monomer

K448-BIO consists of the 448 N-terminal amino acids of the *Drosophila* kinesin α -chain followed by linker amino acids Ser-Ser, followed by the 87 C-terminal amino acids of the BCCP subunit of *E. coli* acetyl-CoA carboxylase. Similarly, K401-BIO contains the 401 N-terminal amino acids of the kinesin α -chain, linker Arg-Lys-Ala, and the 87 C-terminal amino acids of BCCP; and K340-BIO contains the 340 N-terminal amino acids of the kinesin α -chain, linker Leu-Ala, and the 87 C-terminal amino acids of BCCP. Plasmid construction and protein expression were essentially as described^{10,20}. K340-BIO, K401-BIO and K448-BIO were expressed in an insect cell line using the baculovirus expression vector system²¹. K401-BIO and K340-BIO were also expressed in *E. coli*, giving similar results to those obtained with baculovirus-expressed proteins. K448-BIO and K401-BIO (from baculovirus) were purified on monomer avidin and ion-exchange columns as described²⁰. K340-BIO (baculovirus) was partially purified by gel filtration chromatography essentially as described¹⁰ or, in some experiments, purified to near-homogeneity as for K448-L²⁰; the properties of the two preparations were similar. K401-BIO (*E. coli*) and K340-BIO (*E. coli*) were also partially purified by the gel filtration method¹⁰, with the following modifications: *E. coli* cell pellets were resuspended in the same buffer as baculovirus cell pellets¹⁰, incubated (0 °C, 45 min), quick-frozen, and stored at -80 °C. Lysates were prepared for gel filtration by 3 cycles of thawing at 45 °C and freezing in liquid nitrogen, incubation (30 min, 0 °C) in 1.0–1.5 mg ml⁻¹ RNase A (Sigma, type II-A) and 0.5–0.6 mg ml⁻¹ DNase I (Boehringer), and centrifugation at 10,900g for 20 min and 200,000g for 30 min. ATPase specific activities²⁰ of purified enzyme preparations at 1.0 mM ATP and 0.2 mg ml⁻¹ microtubules at 25 °C: K448-BIO, 7 s⁻¹ per head; K401-BIO, 17 s⁻¹ per head; K340-BIO, 2 s⁻¹ per head.

* Diffusion coefficients ($D_{20,w}$; mean ± s.d.) were determined by chromatography at 4 °C on a Superose-6 (Pharmacia) column²⁰; each measurement was repeated 3–5 times in 50 mM imidazole-Cl⁻ (pH 6.7), 50, 200 or 250 mM NaCl, 4 mM MgCl₂, 2 mM EGTA, 50 nM ATP and 10 mM β -mercaptoethanol. $D_{20,w}$ were calculated from a linear fit to $\ln(D_{20,w})$ versus elution volume of standard proteins (Boehringer): horse spleen apoferritin ($D_{20,w}$ = 3.61 × 10⁻⁷ cm² s⁻¹), rabbit muscle aldolase ($D_{20,w}$ = 4.63 × 10⁻⁷ cm² s⁻¹), bovine serum albumin ($D_{20,w}$ = 6.15 × 10⁻⁷ cm² s⁻¹), and hen egg albumin ($D_{20,w}$ = 7.8 × 10⁻⁷ cm² s⁻¹). Elution volumes were determined from nitrocellulose blots stained for biotinylated proteins¹⁰ or from absorbance at 280 nm (ref. 20).

† Sedimentation coefficients ($s_{20,w}$; mean ± s.d. ($N=3$)) were determined by centrifugation of cell lysates and standard proteins in a 12-ml 5–20% sucrose gradient in 50 mM imidazole-Cl⁻ (pH 6.7), 200 or 250 mM NaCl, 4 mM MgCl₂, 2 mM EGTA, 50 nM ATP, and 10 mM β -mercaptoethanol in an SW40.1 rotor (Beckman) at 39,000 r.p.m. for 30–36 h at 4 °C. The position of the standard proteins was determined by SDS-polyacrylamide gel electrophoresis; the position of the kinesin derivatives was determined by nitrocellulose blotting. Sedimentation coefficients were determined by a linear fit to $s_{20,w}$ of the standard proteins rabbit muscle aldolase (Boehringer, 7.35S), bovine serum albumin (Boehringer, 4.31S) and chicken egg white lysozyme (Sigma, 1.91S) versus volume.

‡ Partial specific volumes ($\bar{v}$) were calculated from the predicted amino-acid composition of the proteins²².

§ Molecular masses were calculated according to: $M_r = s_{20,w}RT/(1 - \bar{v}\rho)D_{20,w}$, where $R = 8.314 \times 10^7$ erg K⁻¹ mol⁻¹, $T = 293$ K, and ρ is the density (1.00 g ml⁻¹) of water at 20 °C.

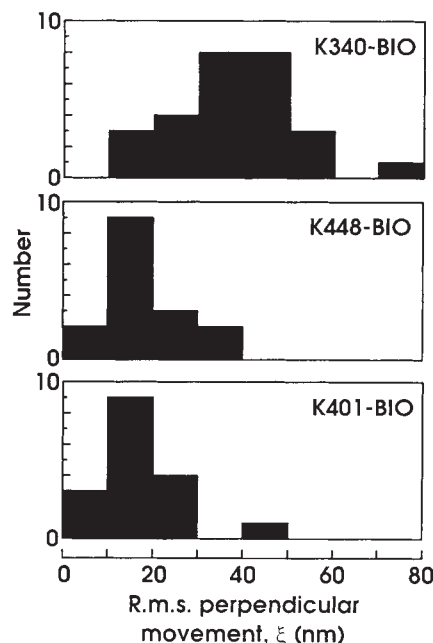
|| Subunit molecular masses were deduced from the predicted amino-acid composition of the proteins.

BIO molecule added per bead, we did not observe motility. This result can be explained if: (1) an individual one-headed molecule is unable to move observable distances continuously along the microtubule, or (2) the average number of active K340-BIO molecules bound to the beads is substantially lower than one. Therefore, we do not yet know whether individual one-headed molecules can move continuously along a microtubule.

Intact kinesin may move using a 'hand-over-hand' mechanism (Fig. 3a), in which one head remains tightly bound to the microtubule while the other detaches from the microtubule and moves forwards^{6–8}. Such a mechanism accounts for the ability of single enzyme molecules to take many steps without being released from the microtubule^{6,7,16}, to track protofilaments² and to sustain piconewton forces (see ref. 16, for example) during move-

FIG. 2 Extent of motion perpendicular to the microtubule axis in motility assays on kinesin derivatives attached to 0.1- μ m diameter beads. Each histogram shows the distribution of r.m.s. perpendicular movement (ξ) values observed for beads driven by the indicated kinesin derivative. For each bead, ξ was computed as the root-mean-square perpendicular displacement between every 2 points in the trajectory that were separated by 200 ± 25 nm in the direction parallel to the microtubule axis. For K340-BIO the distribution mean is 38.9 ± 13.5 (s.d.) nm. For the combined K448-BIO and K401-BIO distributions, the mean is 18.5 ± 9.3 nm.

METHODS. To determine microtubule orientation, we measured the position of a video cursor superimposed at several locations along the microtubule image and fit these with a line (by minimizing the square of the displacements of the measured positions perpendicular to the fit line). We then calculated the projections of the moving bead data parallel and perpendicular to the microtubule orientation¹. A few microtubules (see for example, Fig. 1, trace 6) were detectably curved, leading to some uncertainty in the orientation measurement. In the calculation of ξ , the 200 nm displacement parallel to the microtubule axis was chosen as a value intermediate between short displacements (where instrumentation noise dominates ξ) and long displacements (where errors in determining microtubule orientation dominate ξ). Reported movement velocities (see text) were determined by linear regression of the displacement parallel to the microtubule axis versus time data.



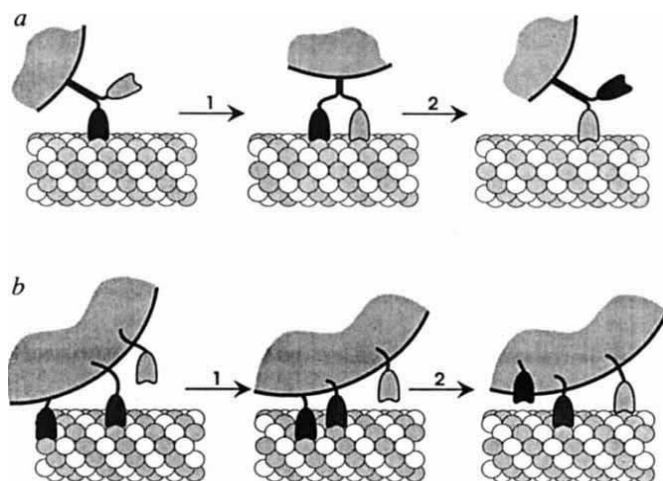


FIG. 3 Cartoons illustrating possible mechanisms for the movement over the microtubule lattice of beads driven by two- and one-headed kinesin derivatives. Horizontal rows of alternating white and shaded circles represent microtubule protofilaments composed of α - and β -tubulin subunits. A portion of a 0.1- μ m diameter spherical bead is shown at the top of each diagram. *a*, One possible 'hand-over-hand' mechanism for the movement of a bead along a single microtubule protofilament by a single two-headed kinesin molecule. The bead is initially attached by a single kinesin head (black). A single 8-nm step¹⁶ is initiated when the free head (shaded) binds to the next tubulin dimer along the same protofilament (step 1), forming a transient intermediate in which both heads are bound. The step is completed when the trailing head releases (step 2). The mechanical properties of the linkage between heads blocks binding to adjacent microtubule protofilaments in step 1 and therefore ensures that the molecule moves along a single protofilament. Beads moved by either single or multiple two-headed molecules will therefore move parallel to protofilaments. Related models, such as those in which the heads move along a pair of adjacent protofilaments, are also consistent with existing data. *b*, Cause of movements perpendicular to the microtubule axis observed for a bead moved by multiple one-headed kinesin molecules located at random positions on the bead surface. Movement is caused by a conformational change in a bound head (black), followed by detachment of the head from the microtubule and subsequent binding to a new lattice site either on the same protofilament (step 1) or a different one (not shown). As the molecules can detach and move to different protofilaments and different groups of molecules can drive movement at different times (step 2), slow random circumferential displacements of the bead occur during motion along the microtubule axis. The circumferential movements are visible as excursions normal to the microtubule axis in the two-dimensional projection seen in the microscope.

ment. Our results (Fig. 3b) verify the prediction of such models, namely that single-headed molecules dissociate from the microtubule during movement and thus do not track. Alternatively, it may not be the monomeric state, but rather the loss or misfolding of a domain requiring residues 341–401, that interferes with K340-BIO protofilament tracking. However, the former interpretation is consistent with the demonstration that a rate-limiting step in the kinesin-microtubule ATPase cycle occurs in a bound state in a two-headed derivative¹⁷, whereas a one-headed derivative displays more complex kinetics¹⁸. The ATPase and tracking results suggest similarities between the movement mechanisms of one-headed kinesin derivatives and that of myosin: (1) a partially rate-limiting step follows a weakly bound state in the actomyosin subfragment-1 mechanism¹⁹, and (2) single heavy meromyosin molecules are not able to maintain continuous association with F-actin during movement⁵. Additional work is required to elucidate the unique structural and mechanistic features of two-headed kinesins that ensure protofilament tracking and prevent detachment of the enzyme molecule from the microtubule during movement. □

Received 15 August; accepted 24 November 1994.

- Gelles, J., Schnapp, B. J. & Sheetz, M. P. *Nature* **331**, 450–453 (1988).
- Ray, S., Meyhofer, E., Milligan, R. A. & Howard, J. J. *Cell Biol.* **121**, 1083–1093 (1993).
- Scholey, J. M., Heuser, J., Yang, J. T. & Goldstein, L. S. B. *Nature* **338**, 355–357 (1989).
- Uyeda, T. Q. P., Warrick, H. M., Kron, S. J. & Spudis, J. A. *Nature* **352**, 307–311 (1991).
- Uyeda, T. Q. P., Kron, S. J. & Spudis, J. A. *J. molec. Biol.* **214**, 699–710 (1990).
- Howard, J., Hudspeth, A. J. & Vale, R. D. *Nature* **342**, 154–158 (1989).
- Block, S. M., Goldstein, L. S. B. & Schnapp, B. J. *Nature* **348**, 348–352 (1990).
- Schnapp, B. J., Crise, B., Sheetz, M. P., Reese, T. S. & Khan, S. *Proc. natn. Acad. Sci. U.S.A.* **87**, 10053–10057 (1990).
- Cronan, J. E. Jr *J. biol. Chem.* **265**, 10327–10333 (1990).
- Berliner, E. et al. *J. biol. Chem.* **269**, 8610–8615 (1994) (see also erratum, 23382).
- Stewart, R. J., Thaler, J. P. & Goldstein, L. S. B. *Proc. natn. Acad. Sci. U.S.A.* **90**, 5209–5213 (1993).
- Chrétien, D. & Wade, R. H. *Biol. Cell* **71**, 161–174 (1991).
- Gelles, J. et al. *Biophys. J.* (in the press).
- Gelles, J., Steuer, E., Schnapp, B. J. & Sheetz, M. P. *J. Cell Biol.* **109**, 157a (1989).
- Qian, H., Sheetz, M. P. & Elson, E. L. *Biophys. J.* **60**, 910–921 (1991).
- Svoboda, K., Schmidt, C. F., Schnapp, B. J. & Block, S. M. *Nature* **365**, 721–727 (1993).
- Hackney, D. D. *J. biol. Chem.* **269**, 16508–16511 (1994).
- Huang, T. G. & Hackney, D. D. *J. biol. Chem.* **269**, 16493–16501 (1994).
- Stein, L., Schwarz, R. Jr, Chock, P. & Eisenberg, E. *Biochemistry* **18**, 3895–3909 (1979).
- Young, E. C., Berliner, E., Mahtani, H. K., Perez-Ramirez, B. & Gelles, J. *J. biol. Chem.* (in the press).
- Summers, M. D. & Smith, G. E. *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures* (Texas Agricultural Experiment Station, College Station, 1987).
- Cohn, E. J. & Edsall, J. T. *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions* 370–381 (Reinhold, New York, 1943).
- Hirokawa, N. et al. *Cell* **56**, 867–878 (1989).
- Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J. & Salemme, F. R. *Science* **243**, 85–88 (1989).
- Staros, J. V., Wright, R. W. & Swingle, D. M. *Analyt. Biochem.* **156**, 220–222 (1986).
- Schnapp, B. J. *Meth. Enzym.* **134**, 561–573 (1986).
- Schafer, D. A., Gelles, J., Sheetz, M. P. & Landick, R. *Nature* **352**, 444–448 (1991).

ACKNOWLEDGEMENTS. We thank A. Bar-Shalom and S. Karki for preliminary studies for this project, Y. Vugmeyster and D. Peisach for assistance, S. Lowey and H. Friedmann for advice, and H. Huxley and S. Lowey for comments on the manuscript. Supported by grants from The Whitaker Foundation and the NIH, by scholar awards (to J. G.) from the Lucille P. Markey Charitable Trust and the Searle Scholars Program, and by a predoctoral fellowship (to E.C.Y.) from the Howard Hughes Medical Institute.

Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA

Jianglin He & Philip Furmanski*

Department of Biology, New York University, 1009 Main Building, Washington Square, New York, New York 10003, USA

LACTOFERRIN, an iron-binding glycoprotein found in high concentrations in human milk and other epithelial secretions¹ and in the secondary (specific) granules of neutrophils², is thought to be responsible for primary defence against microbial infection, mainly as a result of lactoferrin sequestration of iron required for microbial growth³. Many other functions have been attributed to lactoferrin, including immunomodulation and cell growth regulation (reviewed in ref. 4). Some of these functions appear to be at least in part independent of the iron-binding activity of lactoferrin. It also has been consistently observed that lactoferrin interacts avidly with nucleic acids^{5–7}. Lactoferrin enhancement of the activity of natural killer and lymphokine-activated killer cells *in vitro* is inhibited by RNA and DNA⁸. Lactoferrin taken up by K562 human myelogenous leukaemia cells appears in the nucleus where it is bound to DNA⁹. We report here that binding of lactoferrin to DNA occurs under stringent conditions with distinct sequence specificity, and that interaction between lactoferrin and these sequences intracellularly leads to transcriptional activation.

The interaction of lactoferrin (LF) with DNA was first characterized using the quantitative filter binding assay¹⁰. LF was incubated with human DNA, sheared to about 300 base-pair lengths

* To whom correspondence should be addressed.

by sonication. Binding of LF to these preparations was saturable and had a dissociation constant K_d of 3.2×10^{-8} M. In contrast, binding of histone to the same DNA preparations was not saturable and had a K_d of 2.5×10^{-7} M. Although poly(dI-dC):poly(dI-dC) stoichiometrically inhibited the histone reaction, inhibition of LF binding to human DNA required much higher concentrations and was biphasic; in the presence of 25-fold molar excess of poly(dI-dC):poly(dI-dC), all reaction with histone was eliminated but 10% of input genomic DNA still bound to LF. Addition of up to 20 mg ml^{-1} bovine serum albumin, which reduces nonspecific binding of DNA to proteins but has little effect on interaction of DNA with sequence-specific binding proteins¹¹, eliminated binding of DNA to histone but had only a modest effect on the DNA-LF binding.

Using the conditions established for high-affinity binding of LF to DNA, we determined whether the binding reaction exhibits sequence specificity or preference using the CASTing method¹²⁻¹⁴. For this, LF was incubated under stringent conditions with a synthetic polynucleotide made up of amplification and cloning sequences flanking a random sequence of 30 base pairs. LF-DNA complexes were isolated by immunoprecipita-

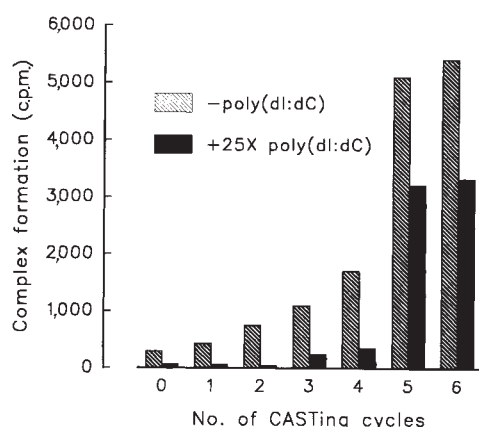


FIG. 1 Enrichment for LF DNA-binding sequences using the CASTing method^{23,24}. Random oligonucleotides were incubated with LF under stringent conditions, the complex isolated by immunoprecipitation with monoclonal anti-LF, dissociated, and the DNA isolated and amplified by PCR reaction. DNA was tested for binding to LF in the presence and absence of poly(dI-dC):poly(dI-dC) as nonspecific inhibitor, and then cycled again through the binding and amplification steps.

METHODS. An oligonucleotide was synthesized consisting of (from 5' end) sites for restriction enzymes *XhoI*, *BamHI* and *EcoRI*, 30 bases of random sequence, followed by sites for restriction enzymes *XbaI*, *HindIII* and *Sall*. Oligonucleotides were made double-stranded using Taq DNA polymerase and a primer complementary to the 5' sequence. All oligonucleotides were confirmed by direct sequence determination. Purified double-stranded oligonucleotide (5 µg) was incubated with 0.5 µg LF (Sigma) in 20 µl binding buffer (50 mM HEPES buffer (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 10 µM ZnSO₄, 2 mM dithiothreitol, 1 mM EDTA, 5% DMSO and 10% glycerol) plus 2 µg poly(dI-dC):poly(dI-dC) and 10 mg ml⁻¹ BSA. After incubation for 30 min at 37 °C, 10 µl of magnetic beads coated with rabbit anti-mouse IgG (Dyna) and monoclonal anti-human lactoferrin H10 (ref. 10) was added and the mixture incubated for 1 h at room temperature. The mixture was diluted with 500 µl isotonic saline containing 0.5% Nonidet P-40 and 0.1% BSA, the beads were recovered by magnetic separation and washed three times with the same buffer and then once with PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin and 200 µM of all 4 deoxynucleotide triphosphates). The beads were resuspended in 22.5 µl PCR buffer, plus 1 µl of a 7.4 µM solution of each primer, and heated at 100 °C for 5 min to dissociate DNA from the complex. Taq polymerase was then added (2.5 units) and the mixture subjected to 16 cycles of heating to 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. The reaction product was purified with a SpinBind Unit (FMC Corp.) and quantified by spectrometry. Amplified DNA (5 µg) was used to initiate the next CASTing cycle or labelled and used in the binding assays.

TABLE 1 Mutational analysis of the consensus DNA-binding sequences of LF

Designation	Sequence	Inhibition of DNA binding (%)	CAT activity (c.p.m.)
Wild type	G G C A C T T G C	97.1	2,597
GmT	G T C A C T T G C	91.7	2,133
CmA	G G A A A T T G C	55.1	1,534
GmC	C G C A C T T G C	47.4	1,054
CmG	G G C A G T T G C	13.0	215
CTdmGG	G G C A G G T G C	10.2	176
SCRBL	G C C A G T T G C	11.3	234

DNA of the indicated sequences (flanked by appropriate restriction sequences for cloning) were synthesized and used to inhibit binding of LF to the end-labelled, wild-type consensus sequence. The competitive binding reactions were analysed by gel mobility retardation assay, quantified by densitometry. Results shown are the percentage of binding to the wild-type sequence remaining in the presence of 25-fold molar excess of competitor. Reporter plasmids containing the mutated sequences were constructed and tested for transcriptional activation by iron-saturated LF as described for the wild-type sequence (Fig. 3).

tion with monoclonal anti-human LF, the DNA amplified with the polymerase chain reaction (PCR), purified and then cycled through the binding, purification and amplification steps. As shown in Fig. 1, DNA that preferentially reacted with LF was progressively enriched through the successive cycles of CASTing. In contrast, histone bound DNA from each of the cycles equivalently (not shown).

DNA from cycle 6 was cloned and 64 individual clones isolated and sequenced. The sequences were first analysed for their relationship using a polyphylogenetic alignment program, which suggested their segregation into three major, related groups. Alignment of the sequences in each of the individual groups yielded three robust consensus sequences: sequence 1, GGGACTT(G/A)C; sequence 2, TAGA(A/G)GATCAAA; sequence 3, ACTACAGTCTACA.

One representative clone from each of the three groups of consensus sequences was further analysed for interaction with LF by several methods. Gel-shift analysis demonstrated strong, cooperative binding of the consensus sequences to LF even in the presence of excess poly dI:dC (Fig. 2A). Two shifted bands were consistently observed: one at relatively low LF concentrations and a second, more slowly migrating band, at higher LF concentrations. Quantification of the binding reaction (Fig. 2B) yielded K_d 's of 1.24×10^{-8} M and saturating molar ratios of LF bound to DNA in the complexes of 1.96:1 and 4.02:1, at low and high LF concentrations, respectively, consistent with the observed rate of migration of the bands in the gels. Similar results were obtained for all three consensus sequences. Binding of DNA to iron-saturated LF was slightly, but reproducibly, more avid than to apo-LF. Addition of monoclonal anti-human LF caused a specific supershift, and South-western blotting under a variety of conditions demonstrated specific binding of the isolated DNA sequences to LF (data not shown). DNase I protection assays showed specific interaction between LF and the consensus sequence regions of the probes used (Fig. 2C). The broad region of DNase I protection may be a reflection of the binding of LF multimers to the DNA sequences.

To examine the consequences of LF binding to the sequences identified, reporter constructs were prepared consisting of the consensus binding sites placed upstream of the CAT gene driven by the weak E1b promoter. Constructs were prepared with a single binding site (E21B1) as well as with four tandem sites (E21B4). The constructs were electroporated into K562 cells, chosen because of the previous report that they take up LF through a cell surface receptor and transport it to the nucleus

where it is found in association with DNA⁹. Following transfection, the cells were grown in the presence and absence of apo-LF or iron-saturated LF and assayed for CAT activity. Cells transfected with the construct containing four tandem LF consensus binding sites and incubated in the presence of LF exhibited substantial CAT activity compared with cells incubated without LF or cells transfected with the control plasmids (Fig. 3). Iron-saturated LF was a more active inducer of CAT activity than was apo-LF. Similar results were obtained with reporter constructs containing a single LF binding site, although the total CAT activity obtained was much lower. Strong transcriptional activation was obtained with consensus sequences 1 and 3, but no activity was obtained with sequence 2, tested in either orientation, positioned upstream or downstream of the reporter gene. Sequence 2 also did not reduce expression of the CAT reporter driven by a strong promoter (not shown).

Mutational analysis revealed that activity was diminished most by substitutions in base positions 1, 2 and 5 (G, G and C, respectively) of the wild-type consensus sequence 1 nine-unit oligomer (9-mer) (Table 1). Transcriptional activation with the mutant sequences correlated well with LF binding, as measured in the gel shift assay. A scrambled sequence (SCRBL) with the same composition as wild-type sequence A exhibited little binding and little transactivation. Iron-saturated LF stimulated

in vitro transcription of the wild-type reporter plasmid 2.4 and 8.0-fold in the presence of an extract of K562 cells or K562 extract plus *Drosophila* embryo extract, respectively (measured using the In Vitro Eukaryotic Transcription Kit, Stratagene). No stimulation was observed with the reporter constructs containing sequences to which LF did not bind (data not shown). Finally, transcriptional activation of the reporter plasmid containing LF binding sequence A was obtained in cells cotransfected with expression vectors for intact LF (pLf) and for LF lacking its 5' signal sequence for extracellular secretion (pS⁻Lf; Fig. 3).

Although defined principally by its high-affinity binding of iron, it has been known for some time that LF may have a wide array of functions, some of which are regulatory and cannot be explained only by metal-binding. Thus, LF is a potent activator of natural killer (NK) cells *in vitro* and *in vivo*⁸, which may play a role in antitumour defence¹⁵, and this activity is independent of iron. LF also regulates granulopoiesis¹⁶, antibody-dependent cell-mediated cytotoxicity⁸, cytokine production^{17,18}, and growth of some cells *in vitro*¹⁹. This study suggests that some of these functions may be the result of LF binding to specific DNA sequences and transcriptional activation. Although most studies of the intracellular distribution of LF have shown it to be confined to the cytoplasm, usually in granules or secretory vesicles, these analyses have been done principally on cells producing LF. In contrast, others have shown LF uptake by cells and, in some

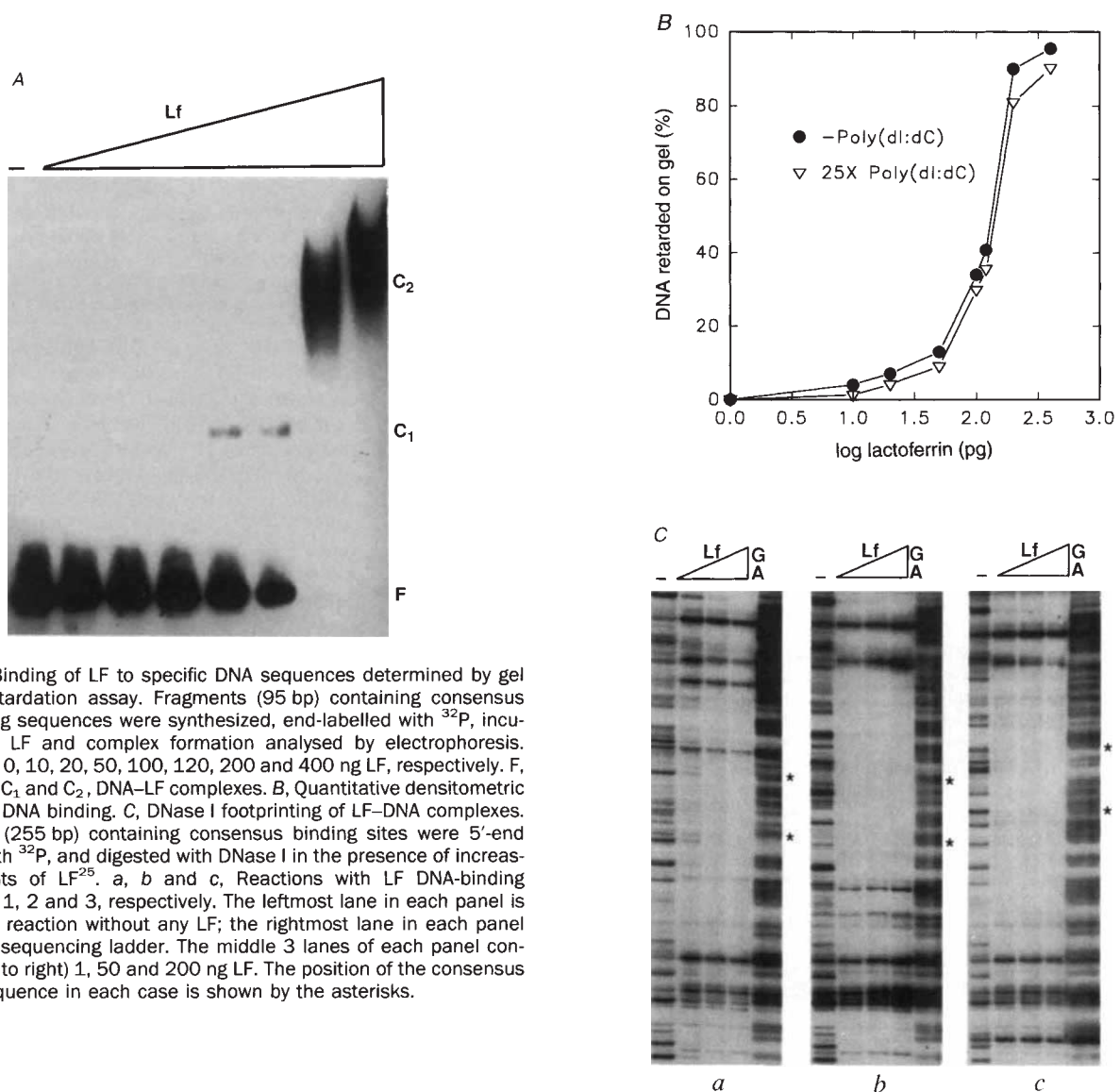


FIG. 2 A, Binding of LF to specific DNA sequences determined by gel mobility retardation assay. Fragments (95 bp) containing consensus DNA-binding sequences were synthesized, end-labelled with ³²P, incubated with LF and complex formation analysed by electrophoresis. Lanes 1–8: 0, 10, 20, 50, 100, 120, 200 and 400 ng LF, respectively. F, free probe; C₁ and C₂, DNA–LF complexes. B, Quantitative densitometric analysis of DNA binding. C, DNase I footprinting of LF–DNA complexes. Fragments (255 bp) containing consensus binding sites were 5'-end labelled with ³²P, and digested with DNase I in the presence of increasing amounts of LF²⁵. a, b and c, Reactions with LF DNA-binding sequences 1, 2 and 3, respectively. The leftmost lane in each panel is the control reaction without any LF; the rightmost lane in each panel is the G/A sequencing ladder. The middle 3 lanes of each panel contained (left to right) 1, 50 and 200 ng LF. The position of the consensus binding sequence in each case is shown by the asterisks.

instances, localization to the nucleus^{9,12,20,21} where it was reported to be bound to DNA⁹. Although LF does not possess a canonical nuclear localization sequence, the N terminus of the protein does exhibit a highly basic region analogous to the SV40 nuclear localization signal¹³ (although this LF region recently has been shown to resemble the arginine-rich, receptor-binding sequence in apolipoprotein E¹⁴). Thus, LF released by neutrophils at the site of infection to sequester iron required for microbial growth may be taken up by target cells such as NK cells or macrophages, already known to be responsive to LF^{8,15}, in which genes are activated to mount a second level of defence against the infecting organisms. Target gene activation may occur in bacterial cells, some of which produce siderophores that retrieve bound iron from LF²². The results also suggest the existence of a new class of transcriptional activators, those secreted by one cell and taken up by a second target cell in which they are transported to the nucleus and directly activate gene expression. Although many cell-signalling and communication molecules are recognized, all those previously described activate multistep intracellular messenger pathways that themselves produce the proximal activators of gene expression. Direct gene activation from an extracellular signalling molecule, through binding to DNA, has not been previously reported, to our knowledge. □

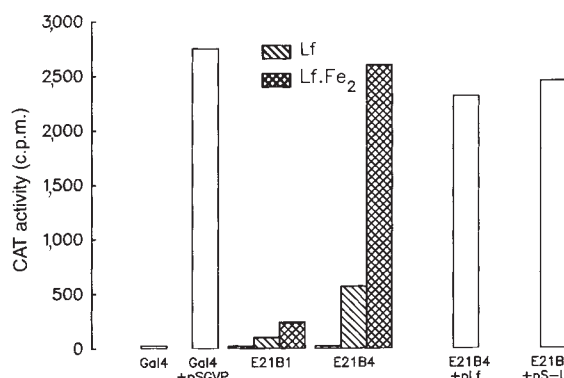


FIG. 3 Activation of CAT reporter gene expression by LF and iron-saturated LF. Transcriptional activation was determined by measuring CAT activity from reporter constructs containing 1 (E21B1) or 4 (E21B4) copies of consensus LF DNA-binding sites transfected into K562 cells grown in the presence or absence of LF and iron-saturated LF at $10 \mu\text{g ml}^{-1}$. Cells were collected 48 h after transfection and cell extracts assayed for CAT activity²⁶. All determinations were normalized to the activity of cotransfected pCMV β Gal²⁷.

METHODS. The K562 cell line was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Cells in late log phase were washed with serum-free medium and resuspended to a density of 1.4×10^7 per ml. For each transfection, 300 μl of cell suspension was mixed with 100 μl solution of DNA in RPMI 1640 (10 μg phenol extracted, sonicated salmon sperm DNA as carrier, 10 μg pCMV β Gal as internal control and the indicated amount of other plasmic DNA). Cells were transfected by electroporation (IBI Gene Zapper), at 100 μF capacitance, 400 V and 400 Ω wave controller resistance. About 10% transfection efficiency was obtained under these conditions as determined by cytochemical analysis of control β -galactosidase expression from pCMV β Gal. Following electroporation, cells were washed once with RPMI 1640 and incubated for 1 h at 37 °C. Iron-saturated LF or apo-LF were then added to a final concentration of $10 \mu\text{g ml}^{-1}$ and 1 h later, FCS was added to a final concentration of 10%. Cells were collected 48 h after electroporation and extracts assayed for CAT activity by phase extraction and scintillation counting²⁶ and assayed for β -galactosidase as described²⁷. All experiments were replicated at least 3 times. Reporter plasmid pG5E1bCAT, containing 5 tandem GAL4 binding sites, and its activator pSGVP (obtained from C. Dang, Johns Hopkins University) were used as controls for activated CAT expression.

Received 3 November; accepted 30 December 1994.

1. Lonnnerdal, B. & Woodhouse, L. *Nutr. Res.* **8**, 853–858 (1988).
2. Spik, G. et al. *Br. Med. J.* **5**, 69–74 (1978).
3. Weinberg, E. D. *Microbiol. Rev.* **42**, 45–66 (1978).
4. Lyer, S. & Lonnnerdal, B. *Eur. J. clin. Nutr.* **47**, 232–241 (1993).
5. Bennett, R. M. et al. *J. clin. Invest.* **71**, 611–618 (1983).
6. Hutchens, T. W. et al. *Pediatr. Res.* **29**, 243–250 (1991).
7. Bennett, R. M. & Davis, J. J. *Immun.* **127**, 1211–1216 (1981).
8. Shau, H. et al. *J. Leuk. Biol.* **51**, 343–349 (1992).
9. Garre, C. et al. *J. Cell Physiol.* **153**, 477–482 (1992).
10. Siebenlist, U. et al. *Cell* **37**, 381–388 (1984).
11. Zhang, X. Y. et al. *Analyt. Biochem.* **201**, 366–370 (1992).
12. Walmer, D. K. et al. *Endocrinology* **131**, 1458–1466 (1992).
13. Meier, U. T. & Blobel, G. *J. Cell Biol.* **111**, 2235–2245 (1990).
14. Ziere, G. J. et al. *J. biol. Chem.* **268**, 27069–27075 (1993).
15. Bezault, J. et al. *Cancer Res.* **54**, 2310–2312 (1994).
16. Broxmeyer, H. E. et al. *Blood Cells* **13**, 31–48 (1987).
17. Zucali, J. R. et al. *Blood* **69**, 33–37 (1987).
18. Zucali, J. R. et al. *Blood* **70**, 191a (1987).
19. Hashizume, S. et al. *Biochim. biophys. Acta* **763**, 377–382 (1983).
20. Green, C. et al. *Proc. Soc. exp. Biol. Med.* **137**, 1311–1317 (1971).
21. Miyauchi, J. & Watanabe, Y. *Cell Tissue Res.* **247**, 249–258 (1987).
22. Weinberg, E. D. *Life Sci.* **50**, 1289–1297 (1992).
23. Funk, W. D. et al. *Molec. cell. Biol.* **12**, 2866–2871 (1992).
24. Rikitake, Y. & Moran, E. *Molec. cell. Biol.* **12**, 2826–2836 (1992).
25. Higuchi, R. et al. *Nucleic Acids Res.* **16**, 7351–7369 (1988).
26. Seed, B. & Sheen, J. Y. *Gene* **67**, 271–278 (1988).
27. An, G. et al. *Molec. cell. Biol.* **2**, 1628–1633 (1982).

ACKNOWLEDGEMENTS. This work was supported by grants from the NIH.

Pre-bending of a promoter sequence enhances affinity for the TATA-binding factor

Jeffrey D. Parvin^{*†}, Richard J. McCormick[‡],
Phillip A. Sharp^{*} & David E. Fisher^{‡§}

^{*} Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, 40 Ames Street, Cambridge, Massachusetts 02139-4307, USA

[‡] Division of Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA

TATA-binding protein (TBP) binds the minor groove of the TATA element with the DNA bent 80° towards the major groove^{1–4}. A constrained minicircle strategy⁵ has been used to test the effect of DNA topology on the affinity of TBP for the TATA element. We report here that TBP bound to DNA which was slightly pre-bent towards the major groove with 100-fold higher affinity than unbent (linear) DNA of identical sequence and 300-fold higher affinity than DNA pre-bent towards the minor groove. Similar discrimination was observed with the holo-TFIID transcription complex. DNA topology, particularly bending, is determined by many factors including chromatin in cells and may, through changes in the affinity of the TATA factor, be important in the control of transcription.

DNA probes were pre-bent by virtue of being constrained in a covalently closed circle, and the direction of bend of the binding site was phased with intrinsically bent sequences (A-tracts). Circular, body-labelled probe contained the intrinsic bend and the immunoglobulin (IgH) promoter sequences so that the centre of the TATA motif was either 31 base pairs (bp) (in phase) or 37 bp (out of phase) from the centre of the nearest A-tract (Fig. 1a). A-tracts bend toward the minor groove⁶; thus the TATA-31 probe constrains the TATA element to be pre-bent towards the minor groove, and the TATA-37 probe pre-bends the TATA element towards the major groove. Because TBP binds DNA bent towards the major groove^{1–3}, DNA pre-bent in this direction might be expected to bind TBP with higher

[†] Present address: Division of Molecular Oncology and Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115, USA.

[§] To whom correspondence should be addressed.

affinity. These exonuclease-resistant circular probes (Fig. 1b) contain 360° of bend distributed along 156 bp, thus the average bend is 2–3° per bp.

The two circular probes or their linear counterparts were incubated with increasing concentrations of TBP and resolved by electrophoretic mobility shift assay (EMSA). TBP bound circular TATA-37 probe at much lower protein concentrations than the other three probes (Fig. 2a). Circular and linear DNA probes lacking a TATA motif were not bound by TBP (data not shown).

Competition experiments also revealed enhanced binding to DNA pre-bent towards the major groove (Fig. 2b). Unlabelled circular TATA-37 DNA efficiently competed for binding to labelled circular TATA-31 probe (compare lane 4 with lanes 1 and 2), whereas competition with either the circular or linear TATA-31 probes was only observed when competitor was present at vast excess.

The binding constants of TBP with IgH promoter probes were calculated from measured kinetic parameters, $k_{\text{off}}/k_{\text{on}}$. EMSA has been successfully used to quantify TBP affinity for linear adenovirus major late promoter (MLP) probe⁷. The truncated TBP molecule used in this study contains the carboxy-terminal

DNA-binding domain for which the crystal structure is known^{1,8,9}. It does not dissociate noticeably during electrophoresis (data not shown; ref. 10). Equilibrium constant measurement by protein titration was not feasible because the association rate of TBP with promoter DNA is slow (see below and ref. 7) and because the complex formed with circular TATA-37 DNA was too stable ($t_{1/2} = 11$ h; see below) to permit establishment of equilibrium. Further, free TBP displayed a solution decay rate constant of $3.0 \times 10^{-4} \text{ s}^{-1}$ under binding conditions ($t_{1/2} = 38$ min; data not shown).

TBP association rates were highest for the circular TATA-37 probe (Fig. 3a). Complexes containing the linear probes accumulated at about equal rates, slightly faster than the circular TATA-31 probe. The bimolecular association rate constants¹¹ (k_{on}) indicate that TBP association with circular TATA-37 probe was 5-fold more rapid than with either linear probe and 7.5-fold more rapid than with the circular TATA-31 probe (Table 1).

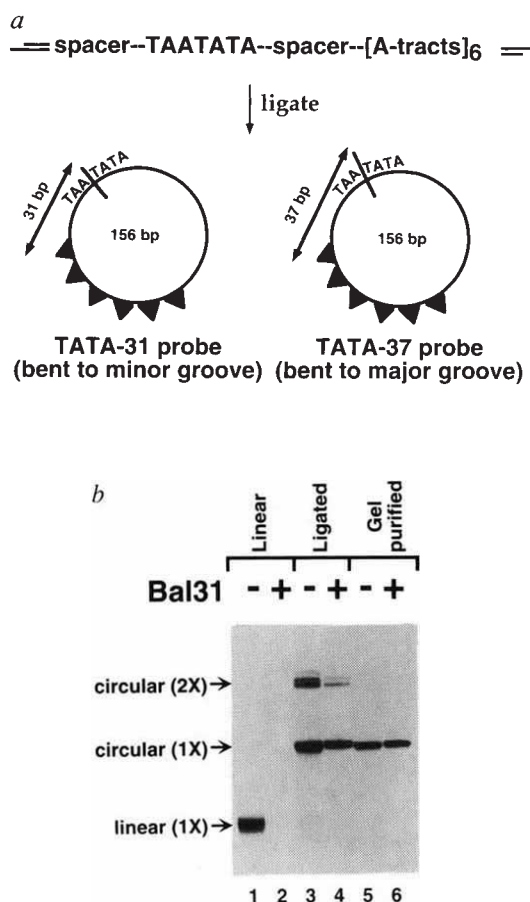


FIG. 1 Minicircle strategy and construction. **a**, Probes were synthesized by polymerase chain reaction (PCR) of plasmids. The total intrinsic bend sequence is a series of six A-tracts, bent to the minor groove. All probes were 156 bp to give an integral number of helical turns in a minicircle. **b**, Linear monomer was prepared by PCR, digestion with restriction enzyme, and gel purification (lanes 1 and 2), ligation (lanes 3 and 4) and gel purification of circular monomer probe (lanes 5 and 6). Exonuclease *Bal31*, which digests DNA with free ends, was incubated with DNAs in lanes 2, 4 and 6.

METHODS. Plasmids were constructed from PCR mutagenesis of plasmid p11T15f (ref. 5) to yield the IgH promoter sequences from -30 to +1 relative to the start site¹⁸ either 3 (31 bp) or 3.5 (37 bp) helical turns from the nearest A-tract when ligated in a minicircle.

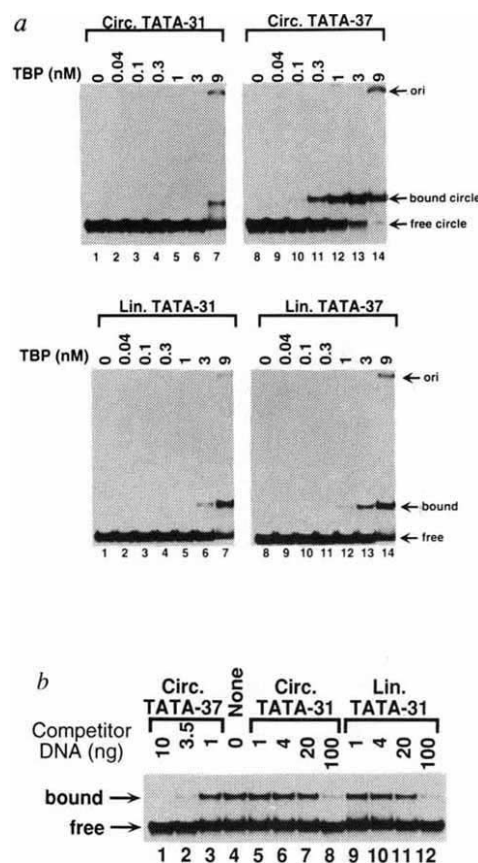


FIG. 2 EMSA of TBP with circular and linear probes. **a**, Differential binding of TBP to probes of varying topology. Binding reactions contained probe alone (lanes 1 and 8) or included TBP increasing in three-fold steps from 40 pM (lanes 2 and 9) to 9 nM (lanes 7 and 14), and were electrophoresed on native gels. In the upper panel, circular probes were used, and in the lower panel the linear probes were used (TATA-31 in lanes 1–7) and (TATA-37 in lanes 8–14). Equilibrium constants could not be determined from these protein titrations (see text). **b**, Competitions with high-affinity circular TATA-37 probe. Competitor DNAs in the indicated quantities, 500 ng poly(dGdC): poly(dGdC) and 9 nM TBP were preincubated for 15 min. circular TATA-37 probe (0.2 ng) was added to each reaction and, after 60 min, samples were subjected to EMSA.

METHODS. Binding reactions (0.015 ml) containing 10 pM probe DNA (in **a**), TBP, 0.06 M KCl, 0.02 M HEPES, pH 7.9, 0.001 M EDTA, 0.005 M MgCl_2 , 0.01 M DTT, 0.15 mg ml^{-1} BSA, and 10% glycerol were incubated for 60 min at 30 °C and resolved on native polyacrylamide gels containing Tris-glycine-EDTA buffer and 0.004 M MgCl_2 . The truncated form of yeast TBP contained the conserved DNA-binding domain (gift from D. I. Chasman; ref. 9). Molarity of TBP determined by titration of probe.

Following initial binding, reactions were quenched and complexes were quantified at times from 2 min to 21 h to measure dissociation rates (Fig. 3b). The circular TATA-37/TBP complex was much more stable than those of either linear probe or the circular TATA-31 probe. The dissociation rate constant for the TBP/circular TATA-37 complex ($t_{1/2} = 11$ h) was 45-fold slower than that for the TBP/circular TATA-31 complex ($t_{1/2} = 15$ min) and about 20-fold slower than that of either TBP/linear DNA complex ($t_{1/2} = 33$ min; Table 1).

To show that the highly stable complex was directly dependent upon DNA topology, circular TATA-37 DNA/TBP complex was linearized by endonuclease cleavage at the time of quenching, and dissociation rates were compared (Fig. 3c). Little dissociation occurred (lanes 7–12) through the 60 min incubation for untreated TBP/circular probe complexes. In contrast, upon linearization of the circular DNA, the dissociation rate increased dramatically such that the half-life was about 30 min (lanes 1–6). Because the same sequence was bound in these linear and

circular probes, linearization must have destabilized the TBP–DNA complex.

The dissociation equilibrium constant (K_d) of TBP for each probe indicated that TBP bound the pre-bent circular TATA-37 probe with 100-fold higher affinity than either linear probe and with 300-fold higher affinity than the pre-bent circular TATA-31 probe. The kinetic and equilibrium parameters determined in this study for the IgH promoter agree with values determined in other studies for linear probes^{7,12}. The k_{on} ($3.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$), k_{off} ($1.1 \times 10^{-4} \text{ s}^{-1}$; $t_{1/2} = 100$ min) and K_d ($3.2 \times 10^{-10} \text{ M}$) for TBP binding a linear MLP probe⁷ fitted with the values determined in this study because the MLP, with its optimal TATA motif, binds with higher affinity than the linear IgH probes, yet pre-bent IgH probe has the highest affinity of all.

HeLa cell-derived holo-TFIID complex containing TBP and associated factors displayed a similar binding preference for DNA pre-bent to the major groove. Using agarose gel

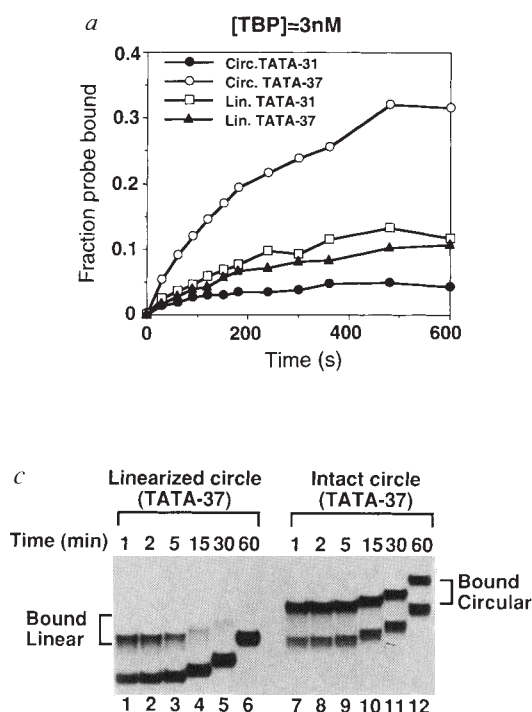
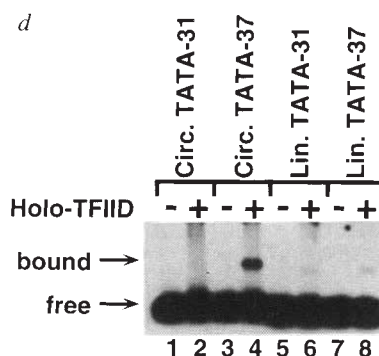
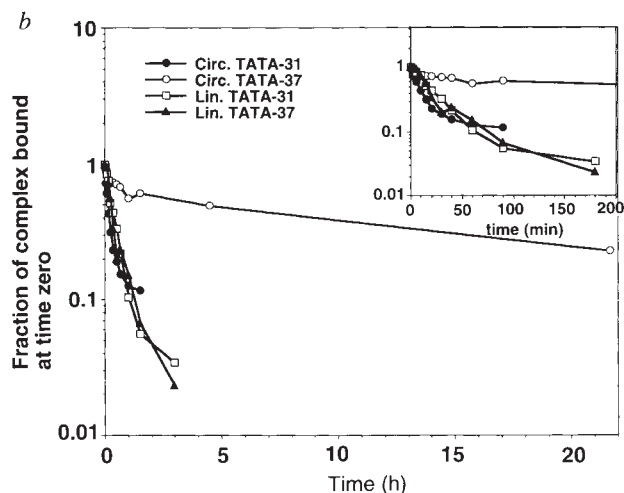


FIG. 3 Determination of kinetic parameters for TBP binding minicircle probes. **a**, Example of the association rate determination. Binding reactions were sampled at the indicated times, quenched by addition of poly(dIdC): poly(dIdC), a specific inhibitor of TBP binding¹⁹, and immediately subjected to EMSA. Gel profiles were quantified by phosphorimager analysis, and k_{on} was determined from the linear portion of the curve (first 3 min in above example) using the bimolecular association rate equation¹¹. Probes were: circular TATA-31 (filled circle); circular TATA-37 (open circle); linear TATA-31 (open square); and linear TATA-37 (filled triangle). **b**, Dissociation rate constant determination. Binding reactions (30 min) were quenched by addition of poly(dIdC): poly(dIdC), incubated for indicated times at 30 °C, subjected to EMSA, and the resulting profile was quantified by phosphorimager analysis. For each probe, binding was normalized to the amount of complex at time zero. Data were omitted if the shifted complex was less than twice background (visually blank). The dissociation rate constant, k_{off} , was determined from slopes of the curves. Inset has early time points expanded. **c**, Linearization of circles destabilizes pre-formed complexes. Circular TATA-37 probe was incubated with TBP, quenched by addition of poly(dIdC), and ClaI, which linearizes the minicircle, was added to half of the reaction (lanes 1–6) and half was undigested (lanes 7–12). At indicated times after addition of enzyme, the reaction was sampled and loaded on a running native gel. The staggered appearance is due to differing times of electrophoresis. **d**, Holo-TFIID complex shows same topological preference as does the TBP subunit. Binding reactions con-



taining each of the four probes with the large holo-TFIID complex were subjected to electrophoresis in agarose gels¹³. Probes were: circular TATA-31 (lanes 1 and 2); circular TATA-37 (lanes 3 and 4); linear TATA-31 (lanes 5 and 6); and linear TATA-37 (lanes 7 and 8). TFIID was included in lanes 2, 4, 6 and 8.

METHODS. **a–c**, The association rate constant equation in protein excess reduces to:

$$\frac{1}{[\text{TBP}]} \ln \frac{[\text{probe}]}{[\text{probe}] - [\text{complex}]} = k_{on}t$$

The expression on the left side of the equation was plotted against time, and the slope, k_{on} , was calculated using the least-squares method provided in CricketGraph software. The dissociation rate constant was calculated from the equation:

$$\ln \frac{[\text{complex}]}{[\text{complex}]_0} = -k_{off}t$$

TABLE 1 Binding constants for TBP and each probe

	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_d (M)
Circular TATA-37	$6.2 \pm 3.3 \times 10^5$	$1.7 \pm 0.1 \times 10^{-5}$	2.7×10^{-11}
Circular TATA-31	$8.1 \pm 4.7 \times 10^4$	$7.6 \pm 2.0 \times 10^{-4}$	9.4×10^{-9}
Linear TATA-37	$1.3 \pm 0.4 \times 10^5$	$3.1 \pm 0.8 \times 10^{-4}$	2.4×10^{-9}
Linear TATA-31	$1.2 \pm 0.2 \times 10^5$	$3.8 \pm 0.6 \times 10^{-4}$	3.2×10^{-9}

The association, k_{on} , and dissociation, k_{off} , rate constants were determined in three or four separate experiments with TBP concentrations ranging from 1 to 6 nM. (Linear TATA-31 k_{off} is from two experiments.) The standard error of the mean is indicated. The dissociation equilibrium constant, K_d , was determined by dividing k_{off} by k_{on} .

electrophoresis¹³, TFIIID binding to circular TATA-37 probe was most efficient (Fig. 3d, lane 4) whereas circular TATA-31/TFIIID was undetectable (lane 2), and complexes containing the linear counterparts were reproducibly present, but weaker (lanes 6 and 8).

For probes with identical binding sequences, TBP affinity varied up to 300-fold depending upon only slight pre-bending of the TATA box. This method⁴ produces a phased DNA bend due to A-tracts and circularization of short DNA fragments. If the DNA is uniformly bent in the circular conformation, the magnitude of the pre-bend is small (16–20° for 7 bp) as compared to the 80° DNA bend in TBP–DNA co-crystals¹³. If DNA were bent to a greater extent, then the observed 300-fold preference for pre-bent DNA may substantially underestimate the total effect. Importantly, this suggests that even modest constraints imposed by a system may significantly impact affinity and affect

transcription regulation. Altering the architecture of promoter DNA may be achieved by, for example, activators and HMG-box proteins which bend the DNA^{14,16}, superhelical turns created by topoisomerases, or through wrapping DNA around nucleosomes. When histones are properly phased on promoter sequences, TBP in the presence of the SWI protein complex can bind in a phase-dependent fashion¹⁷. Sequence-independent alteration in TBP affinity may thus provide a link between chromatin structure and regulated gene expression. □

Received 13 October; accepted 28 December 1994.

- Kim, Y., Geiger, J. H., Hahn, S. & Sigler, P. B. *Nature* **365**, 512–520 (1993).
- Kim, J. L., Nikolov, D. B. & Burley, S. K. *Nature* **365**, 520–527 (1993).
- Kim, J. L. & Burley, S. K. *Nature struct. Biol.* **1**, 638–653 (1994).
- Horikoshi, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1060–1064 (1992).
- Kahn, J. D. & Crothers, D. M. *Proc. natn. Acad. Sci. U.S.A.* **89**, 6343–6347 (1992).
- Koo, H. S., Drak, J., Rice, J. A. & Crothers, D. M. *Biochemistry* **29**, 4227–4234 (1990).
- Hoopes, B. C., LeBlanc, J. F. & Hawley, D. K. *J. biol. Chem.* **267**, 11539–11547 (1992).
- Kim, J. L. & Burley, S. K. *Nature struct. Biol.* **1**, 621–637 (1994).
- Chasman, D. I., Flaherty, K. M., Sharp, P. A. & Kornberg, R. D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8174–8178 (1993).
- Kuddus, R. & Schmidt, M. C. *Nucleic Acids Res.* **21**, 1789–1796 (1993).
- Riggs, A. D., Bourgeois, S. & Cohn, M. J. *molec. Biol.* **53**, 401–417 (1970).
- Hahn, S., Buratowski, S., Sharp, P. A. & Guarente, L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5718–5722 (1989).
- Lieberman, P. M. & Berk, A. J. *Genes Dev.* **8**, 995–1006 (1994).
- Giese, K., Cox, J. & Grosschedl, R. *Cell* **69**, 185–195 (1992).
- Du, W., Thanos, D. & Maniatis, T. *Cell* **74**, 887–898 (1993).
- Bazett-Jones, D. P., LeBlanc, B., Herfort, M. & Moss, T. *Science* **264**, 1134–1137 (1994).
- Imbalzano, A. N., Kwon, H., Green, M. R. & Kingston, R. E. *Nature* **370**, 481–485 (1994).
- Parvin, J. D., Timmers, H. T. M. & Sharp, P. A. *Cell* **68**, 1135–1144 (1992).
- Starr, D. B. & Hawley, D. K. *Cell* **67**, 1231–1240 (1991).

ACKNOWLEDGEMENTS. We thank D. I. Chasman and B. M. Shykind for kindly providing pure TBP and holo-TFIIID, J. Kahn for helpful advice and the A-tract plasmid and S. Buratowski, J. Kim and R. E. Kingston for reading the manuscript. This work was supported in part by a grant from the Fundacion Internacional Jose Carreras to D.E.F. and from the NIH to P.A.S. J.D.P. is a special fellow of the Leukemia Society of America.

GUIDE TO AUTHORS

PLEASE follow these guidelines so that your manuscript may be handled expeditiously.

Nature is an international journal covering all the sciences. Contributors should therefore bear in mind those readers who work in other fields and those for whom English is a second language, and write clearly and simply, avoiding unnecessary technical terminology. Space in the journal is limited, making competition for publication severe. Brevity is highly valued. One printed page of *Nature*, without display items, contains about 1,300 words.

Manuscripts are selected for publication according to editorial assessment of their suitability and reports from independent referees. They can be sent to London or Washington and should be addressed to the Editor. Manuscripts may be dealt with in either office, depending on the subject matter, and will where necessary be sent between offices by overnight courier. High priority cannot be given to pre-submission enquiries; in urgent cases they can be made in the form of a one-page fax. All manuscripts are acknowledged on receipt but fewer than half are sent for review. Those that are not reviewed are returned as rapidly as possible so that they may be submitted elsewhere without delay. Contributors may suggest reviewers; limited requests for the exclusion of specific reviewers are usually heeded. Manuscripts are usually sent to two or three reviewers, chosen for their expertise rather than their geographical location. Manuscripts accepted for publication are processed from the London office.

Nature requests authors to deposit sequence and crystallographic data in the databases that exist for this purpose.

Once a manuscript is accepted for publication, contributors will receive proofs in about 4 weeks. *Nature's* staff will edit manuscripts with a view to brevity and clarity, so contributors should check proofs carefully. Manuscripts are generally published 2–3 weeks after receipt of corrected proofs. *Nature* does not exact page charges. Contributors receive a reprint order form with their proofs; reprint orders are processed after the manuscript is published and payment received.

Categories of paper

Review Articles survey recent developments in a field. Most are commissioned but suggestions are welcome in the form of a one-page synopsis addressed to the Reviews Coordinator. Length is negotiable in advance.

Progress articles review particularly topical developments for a nonspecialist readership. They do not exceed 4 pages in length. Suggestions may be made to the Reviews Coordinator in the form of a brief synopsis.

Articles are research reports whose conclusions are of general interest and which are sufficiently rounded to be a substantial advance in understanding. They should not have more than 3,000 words of text (not including figure legends) or more than six display items and should not occupy more than five pages of *Nature*.

Articles start with a heading of 50–80 words written to advertise their content in general terms, to which editors will pay particular attention. The heading does not usually contain numbers, abbreviations or measurements. The introduction to the study is contained in the first two or three paragraphs of the article, which also briefly summarizes its results and implications. Articles have fewer than 50 references and may contain a few short subheadings.

Letters to Nature are short reports of outstanding novel findings whose implications are general and important enough to be of interest to those outside the field. Letters should have 1,000 or fewer words of text and four or fewer display items. The first paragraph describes, in not more than 150 words and without the use of abbreviations, the background, rationale and chief conclusions of the study for the particular benefit of non-specialist readers. Letters do not have subheadings and contain fewer than 30 references.

Commentary articles deal with issues in, or arising from, research that are also of interest to readers outside research.

News and Views articles inform nonspecialist readers about new scientific advances, sometimes in the form of a conference report. Most are commissioned but proposals can be made in advance to the News and Views Editor.

Scientific Correspondence is for discussion of topical scientific matters, including those published in *Nature*, and for miscellaneous contributions. Priority is given to letters of fewer than 500 words.

Preparation of manuscripts

All manuscripts should be typed, double-spaced, on one side of the paper only. An original and four copies are required, each accompanied by artwork. If photographs are included, five sets of originals are required; for line drawings, one set of originals and four good-quality photocopies are acceptable. Reference lists, figure legends and tables should all be on separate sheets, all of which should be double-spaced and numbered. Three copies of relevant manuscripts in press or submitted for publication elsewhere should be included with submitted manuscripts, clearly marked as such. Five copies of revised and resubmitted manuscripts, labelled with their manuscript numbers, are required, together with five copies of a letter detailing the changes made.

Titles are brief and simple. Active verbs, numerical values, abbreviations and punctuation are to be avoided. Titles should contain one or two key words for indexing purposes.

Artwork should be marked individually and clearly with the author's name and, when known, the manuscript number. Ideally, no figure should be larger than 28 by 22 cm. Figures with several parts are to be avoided and are permitted only if the parts are closely related, either experimentally or logically. Unlettered originals of photographs should be provided. Suggestions for cover illustrations, with captions and labelled with the manuscript number, are welcome. Original artwork is returned when a manuscript cannot be published.

Protein/nucleotide sequences should ideally be in the three-letter and not the single-letter code for amino acids. One column width of *Nature* can accommodate 20 amino acids or 60 base pairs.

Colour artwork. A charge of £500 per page is made as a contribution towards the cost of reproducing colour figures. Inability to pay these costs will not prevent publication of essential colour figures if the circumstances are explained. Proofs of colour artwork may be sent to contributors under separate cover from their galley proofs.

Figure legends should not exceed 300 words and ideally should be shorter. The figure is described first, then, briefly, the method. Reference to a method published elsewhere is preferable to a full description. Methods are not described in the text.

References are numbered sequentially as they appear in the text, followed by those in tables and finally by those in figure legends. Only papers published or in the press are numbered and included in the reference list. All other forms of reference should be cited in the text as a personal communication, manuscript submitted or in preparation. Text is not included in reference lists. References are abbreviated according to the *World List of Scientific Periodicals* (Butterworths, London, 1963–65). The first and last page numbers are included; reference to books includes publisher, place and date.

Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided whenever possible and, if used, defined. Footnotes are not used in the text.

Acknowledgements are brief and appear after the reference list; grant and contribution numbers are not allowed.

Supplementary Information is material relevant to Articles or Letters which cannot, for lack of space, be published in full, but which is available from *Nature* on request.

Submission. Manuscripts can be sent to the Editor at 4 Little Essex Street, London WC2R 3LF, UK or at 1234 National Press Building, Washington, DC 20045, USA. A telephone and fax number should be included. Manuscripts or proofs sent by air courier to London should be declared as 'manuscripts' and 'value \$5' to prevent the imposition of import duty and value-added tax.

Information management

Take this exit on the information superhighway to find software for a digital notary system, automated curve fitting, ELISA data analysis and graphical and statistical analysis.

SURETY Technologies has introduced **digital notary software** to freeze the contents of digital documents and electronic records by affixing a secure digital time-stamp (*Reader Service No. 100*). This system can be accessed over the Internet on a stand-alone basis for certifying documents as they are created or automatically to certify records as part of daily business processes. The digital notary software creates a unique digital fingerprint which is sent to a Surety-coordinating server via the Internet. The coordinating server mathematically combines the fingerprint with other fingerprints coming in at the same time, forming trees that close at one-second intervals. The server then transmits the verifying data back to the user's computer to certify the document. This information includes a timestamp that, when mathematically combined with the document's digital fingerprint, links the document to the Surety validation record. The entire certification process is said to take only seconds.

IngenyVision has been developed by Ingeny for the **analysis of complex two-dimensional DNA spot patterns** (*Reader Service No. 101*). This Windows-based system uses internal marker spots to guide calibration of the spot patterns to databases containing sets of known positions, for rapid and accurate pattern analysis. Multi-window comparison of up to 16 gel patterns is possible. It can accept TIFF-format images from a scanner, a charge-coupled device camera, a PhosphorImager, and the like. Output scorings files are in standard ASCII format for direct import into spreadsheet or statistical software. Data-bases are available containing x/y coordinates of different spots but also come with extra information about the nature (type of mutation, chromosomal location, etc.) of the spots.

Media Cybernetics has announced the release of the **stage controller plug-in module** for the Image-Pro Plus image-analysis software for Windows (*Reader Service No. 102*). Researchers using either a Prior Scientific or Ludl Electronic Products stage can fully control and automate the movement of the stage from within the Image-Pro Plus application. Tiled image capture is also available allowing users to assemble multiple frames into a single high-resolution image. The stage-control module makes this high-resolution image capture possible using standard low-cost video cam-

eras and frame grabbers. Standard point-and-click functions for stage control include single-step movement in the x , y and z planes, auto-focus, a coordinate-memory record and stage position display. By integrating control of the stage into a macro program users can incorporate any of Image-Pro's 200 additional analysis and processing routines into a fully automated image capture, analysis and data collection process.

Adept Scientific has released Stanford Graphics 3.0, designed to provide technical solutions for **scientific and engineering applications** (*Reader Service No. 103*). This program can link Excel, Lotus 1-2-3 or ASCII files directly to a spreadsheet format which can then be displayed by choosing from one of 171 preformatted



Stanford Graphics 3.0.

types of graphs, including error bar charts, three-dimensional vector plots, colour-mapped surfaces and contours. The software also features on-screen curve and surface fitting, two-dimensional and three-dimensional function visualization, FFTs, data smoothing and over 50 mathematics and statistics functions. The enhancements of this version focus on usability and scientific graphing flexibility including faster data import and faster spreadsheet calculations. A formula imager graphically solves user-defined equations. The export formats for Stanford Graphics 3.0 include encapsulate post script (EPS), tag image file format (TIFF), and computer graphics metafile (CGM). Its graphics import options include AutoCAD, Harvard Graphics and EPS.

Scada software, now available from Anville Instruments, is a modular, menu-driven software package for industrial and

scientific **real-time monitoring, data logging and control** (*Reader Service No. 104*). The software provides a set of monitoring and control functions including data logging; process monitoring with full alarm configuration; real-time displays with trend graphs, bar charts and mimic diagrams; and process control with open- and closed-loop facilities. Capable of monitoring up to 1,000 analog and 3,200 digital inputs, the software concurrently runs up to ten independent data-logging programs, allowing data to be logged and displayed from various input devices and across various time bases. Input channels are individually scaled and titled, with multiple-level and rate-of-change alarms. The real-time graphical trend, bar chart and mimic displays can be 'rolled back' to allow the viewing of previously recorded data without affecting current data scanning and storage. Direct communication into a spreadsheet allows further system flexibility for online calculations, event handling and report generation. This system offers full IBM-PC AT compatibility.

Statistics and graphics

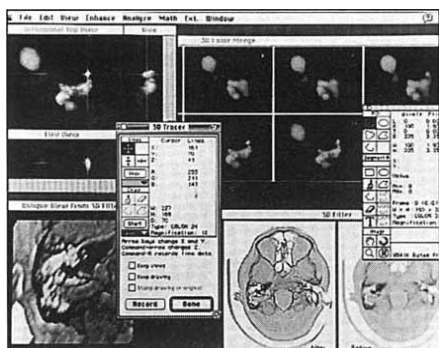
For **automated curve fitting** Jandel Scientific now offers TableCurve 2D (*Reader Service No. 105*). This package offers 32-bit processing and 3,456 linear and nonlinear functions to find the best fit equation for an x/y data set. Equations are ranked according to user-selected best-fit criteria (r^2 , degrees of freedom r^2 , root mean standard error or F). The user can review each of the fitted equations by scrolling through the equation list and viewing the corresponding curve-fit graph. Detailed statistical and precision summaries are also available with each fitted equation to evaluate results further. Multi-tasking is now available for Windows NT users.

Galactic Industries has introduced Grams/3D for **viewing three-dimensional data** from virtually any angle (*Reader Service No. 106*). Data can be tilted, rotated, zoomed and moved in real time using interactive three-dimensional camera effects. Data can be displayed in wire frame, solid and contoured — it can also be 'sliced' in different dimensions to view individual features more closely. This program is designed for the examination of data from a variety of instrumentation including hyphenated techniques (GC-IR and HPLC-DAS), multidimensional spectroscopy (two-dimensional NMR and two-dimensional IR) and spectroscopic

mapping experiments. The manufacturer states that large three-dimensional data sets can be processed and displayed in real-time by coupling Grams/3D with the Grams/386 array-basic programming language. This is said to allow the display of partial-least-squares score plots, correlation plots and many others. This system was designed to utilize the Pentium series processor, while still providing acceptable speed on 486-based systems.

For scientific **graphics and statistical analysis**, DataMost offers StatMost for Windows version 2.0 (*Reader Service No. 107*). This program takes advantage of a multiple-document interface, dynamic data exchange, object linking and embedding, toolbar and toolbox, flexible graphics and a report editor. It can perform statistical analysis (descriptive, ANOVA, regression, non-parametric and correlations), comprehensive numerical computation, time-series analysis, Fourier transform, non-linear parameter estimation and model development. Data can be exchanged with most popular spreadsheet file formats including Lotus 1-2-3, dBase, Quattro Pro, Microsoft Excel and ASCII. StatMost uses direct memory access technology for efficient memory management and algorithms for data editing and sorting, numerical computation, statistical analysis, and model development.

Now offered by Signal Analytics is the IPLab Spectrum extension called 3D, designed specifically for **displaying and analysing three-dimensional data structures** (*Reader Service No. 109*). The package consists of several modules which operate on both greyscale and 24-bit colour image data. Users can create movie-loop



Signal Analytics IPLab Spectrum 3D.

image projections from various points in space allowing users to view spatial relationships among different components of a three-dimensional specimen. A command places images in a two-dimensional array to simplify the publication of three-dimensional image data. Time sequences of multispectral images, such as fluorescence *in situ* hybridization data, can be merged into full-colour image sequences and can be displayed in a movie loop.

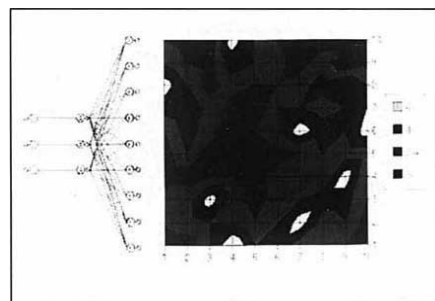
DeltaSoft applications software for the Macintosh provides **data analysis for ELISA applications** (*Reader Service No. 108*). The manufacturer states that all aspects of kinetic and endpoint assays can be controlled with the versatile programming of this software. Microplates can be read one at a time or in series with a single command. This package will read multiple plates, label them sequentially, store them in a single file and display kinetic rate data for all 96 wells simultaneously as it is generated. It can also be programmed to automatically save or report data. Individual wells can be observed with the 'zoom' function to confirm the linear portion of a kinetic rate curve. Negative kinetics can also be analysed where absorbance decreases with time.

■ Data handling and display

Beckman's PeakPro C/S software for **chromatographic analysis** is designed to provide a single source for graphical method development (GMD), chromatogram manipulation and plotting (*Reader Service No. 110*). Client/server architecture permits all graphics tools to be used simultaneously by multiple users. The plot mode accommodates both on/off toggling of the reference graph and plots of 1-100 chromatograms with up to 150,000 points. The operator can set line colour, style and font attributes of the x and y axes, time/voltage data points, grid lines, plot area and page area of the screen. Chromatogram manipulation allows the addition, subtraction, scaling and smoothing of chromatograms.

Perkin-Elmer has introduced UV WinLab software which provides routines for spectroscopic data handling, quantitative analysis, kinetics and instrument validation for the Lambda series of **ultraviolet/visible/near-infrared spectrometers** (*Reader Service No. 111*). The package runs on Microsoft Windows and provides full documentation for meeting ISO 9001 and good laboratory practices. The scan method editor delivers all selections for the scan range, instrument parameters, sampling accessories, sample information and results output. For custom output, the report manager is useful for tailoring hardcopy results. UV WinLab also provides concise procedures for routine quantitative analysis.

Neural Computer Sciences has released a new algorithm for its Windows-based Neudesk neural network, expanding the packages ability to **analyse complex data sets** (*Reader Service No. 112*). The manufacturer states that the algorithm implements Kohonen's concept of competitive learning, which allows a network to adapt to discover patterns and associations in data without supervision. This capability is said to open up new data analysis possibilities for neural



Algorithm allows unsupervised searches.

networks as it does not rely on the presence of correct answers to develop patterns. Applications for the new Kohonen algorithm include: data reduction, image and spectral analysis, the detection of unusual occurrences and real-time alarm monitoring. It is also intended for use in database mining — sifting through large volumes of material to detect grouping and subtle associations. The results of analysis can be processed to produce a topological map of the data, which may then be used to visualize the data and identify patterns or oddities.

Data Transition has released Global Lab Image 3.0 which can accommodate **add-in modules for image analysis software** (*Reader Service No. 113*). Applications include densitometry, pattern recognition, image compression and conversion and image databasing. This version features

ADVERTISEMENT

Astute

Statistics Add-in for Microsoft® Excel

Astute is a new statistical analysis add-in for Microsoft Excel for Windows™.

It is a fast, easy-to-use and powerful application that features 17 commonly used parametric and non-parametric statistical tests.

Advanced data handling even allows missing and non-numeric values to be included within the datasets.

Astute utilises the flexible spreadsheet and charting capabilities of Excel to provide unrivalled output of results that can be fully customised to meet your needs. And by using OLE, you can integrate your results into other applications, such as a word-processing document.

Astute is available for £79 + p&p + VAT. For further information please contact:

DDU Software
The University of Leeds
Old Medical School
Leeds LS2 9JT, UK
Tel: (44) 113 233 4167
Fax: (44) 113 233 5672

Reader Service No.27

new particle measurements, enhanced particle counting and new filters and frame-grabber supports. Users can choose from off-the-shelf modules or they can develop their own. The manufacturer states that Global Lab can be extended to meet the changing needs of scientific users. Add-in modules are said to integrate seamlessly with the base package and share the same user interface. Module developers using Windows can create their own modules using GLIDE (Global Lab image development environment) to accommodate special hardware. GLIDE combines all the features of the base system with an imaging library of algorithms so that developers can create custom modules. The module development standards are an extension of DT-Open Layers and open to anyone.

Cherwell Scientific has announced the release of proFit 4.2, a **data analysis and graphical presentation program** developed by Quantum Soft (*Reader Service No. 114*). This is a program for the investigation, analysis and representation of functions and data. It is designed for a Macintosh interface and features interactive parameter modelling and curve fitting, spreadsheet data management and other programmable options. The proFit 4.2 program is intended for use by physicists, engineers and other scientists requiring all the standard functions of a scientific data analysis and graphing program.

■ Databases

Synopsis has introduced BioCatalysis, a new **chemical reaction database**, which provides information on the use of biomolecules as catalyst in organic synthesis (*Reader Service No. 115*). This system is designed to assist with the use of enzymes and microorganisms in synthesis, both as catalyst for novel processes and as versatile alternatives to traditional methods. The advantages offered by BioCatalysis include chemo-, regio- and enantioselectivity — as well as environmental benefits. The BioCatalysis database offers access to the important information on the subject of biomolecule-mediated organic synthesis and is supplied for use with popular reaction-searching systems, including REACCS, ORAC and ISIS/Host.

Derwent has published a **gene therapy database** from over 1,200 scientific journals, patents from 36 countries and worldwide conference proceedings onto one CD-ROM (*Reader Service No. 116*). The manufacturer states that every development that has been made in gene therapy since the initial findings in 1982 can be located on this database. Subscribers to the database will receive a complete, updated database containing 100–200 new abstracts that identify the latest develop-

ments in gene therapy. Implementation of four classification codes on the database facilitate rapid search of the following subject areas: therapeutic indication, gene transfer system/vector and gene manipulation technique. There is also an additional classification section for legal and ethical issues.

John Libbey Eurotext has developed a **genome interacting database (GID)** (*Reader Service No. 117*). It is a compilation of available knowledge on human genome mapping including Genatlas, Genethon, CEPH and Genset databases. This collection offers 300 Mb of interactive data including 3,000 genes; 900 genetic disorders; 1,100 anonymous D segments; 33,000 YACs; 15,000 oligonucleotides; 17,000 references and 3,000 abstracts from Medline. GID is available in Microsoft Windows and Macintosh versions.

The NIST/EPA/NIH **mass spectral database** is now available in a fully compatible Microsoft Windows version from HD Science (*Reader Service No. 118*). This program is designed to allow access to one of the most widely used mass spectral libraries. It can be used for routine or specialized searches from the user's own work space. Available on diskette or CD-ROM, this program can read various commercial data formats and can be set up to import spectra automatically. The system features a complete 62,250 compound library with over 12,000 selected replicate spectra, incremental compound name search, a similarity search for finding a similar compound if the unknown is not in the library and optimized spectrum-matching algorithms.

■ Miscellaneous

Savant has added capillary electrophoresis (CE) to its **computer-based training line** (*Reader Service No. 119*). This three-module, Windows-based training program teaches the principles of CE and method development. The first module provides an overview and teaches fundamental concepts of free solution capillary electrophoresis (FSCE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MECC) and instrumentation. The second module discusses techniques for detection, sample preparation, buffers, capillaries, method development and an optimal strategy for FSCE. In the third module, micelles, applications, electrolytes, capillary construction and a strategy for method development (with optimization examples for MECC) are covered. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

ADVERTISEMENTS

ProCite

The Software Solution To Managing Bibliographic References

ProCite offers a convenient way to manage, distribute, and print bibliographic references. Automatically generate formatted bibliographies of any type, or create your own electronic catalog of publications, collections, and research information.

PBS, Inc. software is available for Windows, Macintosh, and DOS operating systems, in single and multi-user versions.

Call for a **FREE** mini-version
(313) 996-1580 ext. 95

Reader Service No.28

DNA SEQUENCING

Using an ABI 373 Automated Sequencing System
£60/sample

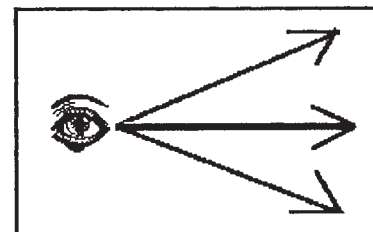
- PCR products - single stranded DNA
- M13 inserts - double stranded DNA
- plasmid inserts - solid phase sequencing
- 300-400 bases of sequence

Please contact us for more information and a preparation protocol

Molecular Medicine Unit

King's College School of Medicine & Dentistry
Tel: 071 346 3126 Fax: 071-733 3877

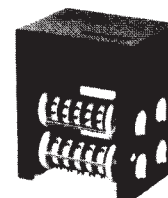
Reader Service No.11



SP140 Photobeam Eye-Tracker
Compact, easy to use, 1msec response.
HVS Image, Ormond Cres. Hampton,
TW12 2TH, UK. Fax +4 4 181 783 1223.
100414.2400@compuserve.com
In the U.S. call 1-800 225 9261

Reader Service No.26

DIABORM® EQUILIBRIUM DIALYSIS SYSTEM



The gentlest method to quantify the binding parameters and thermodynamic data between ligands and biopolymers. Results come closest to the actual values.
Sample volume from 0.2 ml to 5.0 ml.
Up to 20 experiments in parallel.

DIABORM
Geräte
P.O. Box 650126
D-81215 Munich

Reader Service No.12